(3)

AD_____

FEASIBILITY OF HUMAN SKIN GRAFTS ON AN ISOLATED BUT ACCESSIBLE VASCULAR SUPPLY ON ATHYMIC RATS AS A SYSTEM TO STUDY PERCUTANEOUS PENETRATION AND CUTANEOUS INJURY

FINAL REPORT

GERALD G. KRUEGER
LYNN K. PERSHING

JUNE 1986

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-82-C-2214

University of Utah
School of Medicine
Salt Lake City, Utah 84132

88 1 [illegible] 41

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER

2. GOVT ACCESSION NO.

3. RECIPIENT'S CATALOG NUMBER

4. TITLE (and Subtitle)
Feasibility of Human Skin Grafts on an Isolated but Accessible Vascular Supply on Athymic Rats as a System to Study Percutaneous Penetration and Cutaneous Injury

5. TYPE OF REPORT & PERIOD COVERED
Final Report, Sept. 1, 1982 - December 31, 1985

6. PERFORMING ORG. REPORT NUMBER

7. AUTHOR(s)
Gerald G. Krueger, M.D., Principal Investigator

8. CONTRACT OR GRANT NUMBER(s)
DAMD17-82-C-2214

9. PERFORMING ORGANIZATION NAME AND ADDRESS
University of Utah
School of Medicine
Salt Lake City, Utah 84132

10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS
62734A,3M162734A875,BA-334

11. CONTROLLING OFFICE NAME AND ADDRESS
Don E. Schackelford, Contracting Officer's Repr.
Dept. of the Army, US Army Med Res & Dev Command
ATTN: SGRD-RMS, Ft. Detrick, Frederick MD 21701-5012

12. REPORT DATE
June 1986

13. NUMBER OF PAGES
67

14. MONITORING AGENCY NAME & ADDRESS(If different from Controlling Office)

15. SECURITY CLASS. (of this report)
Unclassified

15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)
Approved for public release; distribution unlimited

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)
Human skin, percutaneous absorption, metabolism, model to study human skin in vivo, percutaneous toxicity, microcirculation of skin, nude rat

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)
The objective of this research is to determine the feasibility of grafting human skin onto congenitally athymic (nude) rats and to isolate the grafted human skin as a flap of functional skin onto an isolated, but accessible, vasculature. Thereafter, the proposed system is to be characterized as to structure and function of the skin and, finally, to be validated as a system for studying percutaneous absorption. During year 1, we confirmed that nude rats, contrary to our expectation, are capable of immunologically rejecting human skin. They do not reject allogeneic (rat) skin grafts. Experiments have now demonstrated that

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

a rather bizarre series of events leads to rejection of human skin grafts on nude rats more than 90% of the time. It appears that this rejection process is humorally (antibody) mediated and is directed at antigens that are not necessarily present in normal human skin prior to grafting, but which develop after engraftment. Low-dose cyclosporine at 25 mg/kg/day prolongs engraftment of human skin for more than 90 days after the cyclosporine is discontinued.

We have initiated validation of the flap model system by constructing it as a rat-rat flap and by measuring the percutaneous absorption of [^{14}C]-benzoic acid across grafted and host skin. Absorption characteristics of these two surfaces are very similar. Experiments have also demonstrated that alteration of the cutaneous surface (i.e., increased hydration) alters the percutaneous absorption of [^{14}C]-benzoic acid. To further validate the system, microcirculation has been directly assessed using dermal fluorometry and laser Doppler flow velocimetry. After altering cutaneous circulation by iontophoretical delivery of phenylephrine, we note that absorption of [^{14}C]-benzoic acid through the treated site is considerably altered with peak levels being delayed nearly 10-fold in the phenylephrine-treated area.

To validate the system as a method to study metabolism of topically-applied agents, we have used vidarabine (an anti-viral agent). Data show that flap skin metabolizes vidarabine (ara-A) to an inactive intermediate (ara-H). We also note that ara-H diffuses from the skin back into the diffusion chamber. These validation experiments, as well as the fact that human skin can be maintained on nude rats, suggest that this model system will be a significant tool in defining percutaneous absorption and toxicity of chemical agents.

AD_____

FEASIBILITY OF HUMAN SKIN GRAFTS ON AN ISOLATED BUT ACCESSIBLE VASCULAR SUPPLY ON ATHYMIC RATS AS A SYSTEM TO STUDY PERCUTANEOUS PENETRATION AND CUTANEOUS INJURY

FINAL REPORT

GERALD G. KRUEGER
LYNN K. PERSHING

JUNE 1986

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-82-C-2214

University of Utah
School of Medicine
Salt Lake City, Utah 84132

Summary

The objective of this research was to determine the feasibility of grafting human skin onto congenitally athymic (nude) rats and to isolate the grafted human skin as a flap of functional skin on an isolated, but accessible, vasculature. This was accomplished. Thereafter, the proposed system was characterized as to structure and function of the grafted skin, and validated as a system for studying percutaneous absorption. This report details the progress made during the 3 years of this feasibility project.

Developing a new technology demands change in technique until acceptable rates of success are achieved. During the final year, our success rate of rat-rat or human-rat skin flaps reaching experimental stage stabilized. By the end of the 3rd year, approximately 50-60% of the rat-human flaps reached the experimental stage. Furthermore, the likelihood of a flap being reutilized on second and third occasions increased. Because our technology stabilized, this Final Report details the methodology for generating the flap.

Analysis of the blood flow volume to the flap revealed that blood flow to the flap was approximately 1-2 ml/min. A correlative analysis of the blood flow to the host and grafted sides of the flap demonstrated no significant differences. Analysis of the blood flow to the flap as a function of flap age, as well as the effect of cyclosporine, demonstrated that the blood flow to the flap did not change significantly during the observation period (4 months). Considerable variability was noted in blood flow in any particular flap on a week to week basis. This was not related to administration of cyclosporine, body temperature, or age of the flap. The variability appeared to result from laser Doppler probe placement on the flap surface. Placement on or near large vessels produced greater readings than probe placement away from large vessels. Flap blood flow plays a critical role in percutaneous absorption. Data demonstrated that percutaneous absorption of benzoic acid was proportional to blood flow to the skin. This was confirmed by first noting that rates of absorption on days with high blood flow were higher than those on days with low blood flow, and that this was not related to flap age. In addition, it was noted that percutaneous absorption of benzoic acid through a site that was artificially vasoconstricted with phenylephrine had a considerably different flux profile than skin that was similarly treated with water only as a control. A series of validation studies was conducted comparing the absorption of caffeine across the rat graft and rat host component of a rat-rat skin flap. These results were compared to absorption across the human component of a human-rat skin flap. As a final analysis, in vivo and in vitro absorption of caffeine was determined using the same human skin source. These data demonstrated no essential difference between the absorption profile across rat graft skin and that of rat host skin. However, the amount of caffeine absorbed across rat skin was approximately 10-fold that across human skin. The comparison of human skin, in vivo vs. in vitro, demonstrated dramatic differences. Flux across human skin in vivo occurred much earlier and was much greater than that which occurred in vitro. The large difference in vitro appeared to be due to more extensive binding in the dermis.

In conclusion, this model system met the objectives of the original proposal and will be a significant tool for those groups concerned with kinetics of percutaneous absorption, metabolism of percutaneously applied compounds, and toxic responses generated by topically applied compounds.

Foreword

In conducting the research described in this report, the investigators have adhered to the "Guide for the Care and Use of Laboratory Animals" as prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

TABLE OF CONTENTS

Statement of the Problem

The objective of this research was to determine the feasibility of grafting human skin onto the congenitally athymic (nude) rat and to isolate the grafted human skin as a flap of functional skin on an isolated but accessible vasculature. Once this objective was accomplished, the proposed system was to be characterized as to the structure and function of said skin and, finally, to be validated as a system for studying percutaneous absorption. This is the Final Report of the progress made in pursuit of the foregoing goals between 1 September 1982 and 31 December 1985.

Background

Percutaneous absorption involves a series of sequential transport processes (1). Compounds that are absorbed traverse the stratum corneum, the epidermis, and the papillary dermis until the superficial vasculature (capillary plexus) is reached. At that point, the compound and/or its metabolites either remain in the dermis or are transported to the rest of the body via the circulatory system. An accurate assessment of this dynamic process in skin (animal or human) in the in vivo state, as a function of time, has been unavailable.

The lack of an accurate assessment of in vivo absorption, metabolism, and compartmentalization of topically applied agents within the skin has resulted in an incomplete understanding of the percutaneous absorption process. The quantitative influence of the cutaneous microcirculation on these processes in vivo is unknown. Understanding the local toxic and/or therapeutic effects of agents which are absorbed across and possibly metabolized by the skin before detection in the systemic circulation is critically important to the fields of transdermal drug delivery and cutaneous toxicology. Heretofore, to assess percutaneous absorption of a topically applied agent in human subjects, investigators have had to rely on the amount of a topically applied agent that appeared in the systemic blood, urine, and/or feces. While the amounts appearing in these various body fluids and secretions have been useful for understanding generalities about the kinetics of compounds absorbed across the skin, definitionally they cannot accurately represent local events in the skin. To circumvent the problem of local events, investigators have turned to in vitro models to study percutaneous absorption.

The currently available in vitro models using split-thickness human skin, which are utilized to study percutaneous absorption, have only been partially verified with in vivo analogues (2). The in vitro models are represented by two types, those that use nonviable skin and those that use viable skin with various media support systems. It is assumed, in the latter, that the media support systems represent the normal biologic support systems inherent to mammals. In neither, however, is there an active blood supply to transport the agent or its metabolite from the test site. These systems depend on simple diffusion through the skin into a receiving chamber. Conclusions about absorption and metabolism in such systems, in which tissue viability decreases as the experiment continues, demand confirmation in a viable model with an established functional vasculature. This laboratory has developed an in vivo model system which provides the investigator with functional viable skin with direct access to its supplying vasculature. The system was developed and generated in several phases. First was the creation of skin sandwich flaps, as

suggested by Black and Jederberg (3). Initially, the skin sandwich flap was composed of a rat-rat skin sandwich pedicle on a defined yet accessible vasculature (4) on which a variety of parameters were validated (5-7). The next phase involved the generation of a grafted human-rat skin flap with the same unique vascular characteristics (8). Our current work involves the validation of the flap model for transdermal flux, drug disposition, and skin metabolism studies following topical drug application.

Materials and Methods

Animals

Outbred nude rats have been used because of their depressed immune system (9,10) and because the partial to complete hairlessness of these rats obviates the need for removal of hair with chemicals or clipping, which can result in damage to the stratum corneum. Initial breeding pairs were obtained from the animal production facility of National Cancer Institute (Frederick, MD). This local colony was expanded by mating male rats homozygous for nude with female rats heterozygous for nude in the manner previously described for expansion of colonies of nude mice (11). Typically, experimental rats weigh between 200 and 300 g at the initiation of experiments.

Materials/instruments

Ketamine, 100 mg/kg, injected intraperitoneally (Ketaject, 100 mg/ml, Parke Davis, Morris Plains, NJ), is used to anesthetize the animals for surgery. Additional doses (approximately 10 mg) are administered to sustain anesthesia as needed. Hypovolemia and shock are lessened with the intraperitoneal administration of 3 ml of bacteriostatic sodium chloride (USP, 0.9%, Elkin-Sinns Inc., Cherry Hills, NJ) at the beginning of the experiment. Instruments that are used in the generation of the sandwich flap include: a dermatome (Brown Electro-Dermatome, Model 666, Zimmer Inc., Warsaw, IN), an operating microscope (Model OPMI 6-SD Carl Zeiss, West Germany) equipped with a fiberoptic illuminator (Model 310187, manufactured for Zeiss by Dyonics, Andover, MA), and a Malis bipolar coagulator (Codman and Shurtell Inc., Randolph, MA). Disposable materials that are used include: sutures, 5-0 Ethilon black monofilament nylon with a PS-2 needle (Ethicon Inc., Somerville, NJ) and a 10-0 black monofilament nylon with a TE 70 needle (Davis + Geck Inc., Maranti, PR); Dermiform hypoallergenic knitted tape (Johnson and Johnson Products Inc., New Brunswick, NJ); Kling conforming gauze bandage (Johnson and Johnson Products Inc., New Brunswick, NJ); heparin (sodium injection, USP 1000 USP units/ml, Elkin-Sinns Inc., Cherry Hills, NJ); [^{14}C]-benzoic acid (specific activity 56.8 or 19.3 mCi/mM, New England Nuclear, Boston, MA); Parafilm (American Can Co., Greenwich, CT); Hollisеal skin barrier (Hollister Inc., Libertyville, IL); sterile Vaseline petrolatum gauze (Chesebrough-Ponds Inc., Hospital Products Division, Greenwich, CT); and Webcol sterile alcohol wipes (Kendall Company Hospital Products, Boston, MA).

Blood flow analysis

Blood flow is assessed noninvasively using a laser Doppler velocimeter (LDV; LD 5000 Med Pacific, Seattle, WA) (12-14) or a handheld dermofluorometer, Fluoroscan (Santa Barbara Technology, Santa Barbara, CA) (15,16). Electromagnetic flow probes (type C with a 0.5-mm diameter, Micron Medical, Los Angeles, CA) directly attached to the experimental vessel are used to measure blood flow through the vessels supplying and draining the skin sandwich flap.

Description of the model

The experimental model consists of a skin sandwich which is generated as a flap by grafting a split-thickness skin graft (STSG), human or rat, 0.5 mm in thickness, to the subcutaneous surface of epigastric skin on the abdomen of the rat. The sandwich flap is then isolated with its supplying vasculature, transferred to the dorsum of the rat through a subcutaneous tunnel, and sutured in place. The inferior mediolateral aspect of skin of the rat's abdomen is supplied and drained by the superficial epigastric vessels. Because of the reliability of these anatomical structures, this area has been identified as an area to study circulation in skin on a vascular island, i.e., skin supplied and drained by a defined vasculature (17). More recently, it has been used to develop and improve microsurgical techniques (18). An island skin flap is defined as a piece of living skin isolated and maintained by an independent and defined vasculature. Our sandwich flap is an island flap that has split-thickness skin grafted to its subcutaneous surface. The size of the flap is defined by the anatomy of the vascular bed (19), which can be readily visualized from the undersurface at the time of surgery. In this setting, the dermis of the donor skin and subcutaneous tissue of the host island flap grow together, sandwiching the vessels supplying the flap, the superficial epigastric vessels.

Generation of skin sandwich flap

The island skin sandwich flap is constructed in three stages (Figure 1):

Stage I: Implantation of the STSG skin
Stage II: Lifting the flap from the rat abdomen
Stage III: Isolation of the flap vasculature and translocation to the rat back.

For stage I, STSG are generated using syngeneic rat dorsal skin, or the human skin remnant from abdominoplasty procedure, which is dermatomed to a thickness of 0.5 mm. The STSG is either used immediately or stored in RPMI 1640 + 5% fetal calf serum at 4°C until used for grafting (not longer than 72 hr). The rat is anesthetized and immobilized on its back for the surgery. The sandwich flap is created by implanting a piece of STSG of syngeneic rat skin or human skin under the skin of the inferior lateral abdomen which is supplied and drained, for the most part, by the superficial epigastric vessels. The recipient area is generated by incising the abdominal skin of the rat along three sides and elevating this skin to its caudal base, at the level of the inguinal ligament. The STSG is trimmed to approximate the size of the host recipient area. The STSG is placed in the wound such that the epidermal side faces the abdominal musculature. The overlying epigastric flap is then returned to its normal anatomic position, where the flap of STSG and host abdominal skin are sutured in place (see Figure 1A). A bandage dressing of conforming gauze is applied to provide gentle pressure against the buried sandwich of skin as well as to inhibit the rat from scratching or gnawing at the surgical site. The experience of this laboratory, as well as that of others, demonstrates that this surgical procedure does not lead to necrosis of the host skin; rather, it generates a situation in which the flap receives nearly all of its blood supply from the superficial epigastric vessel (17,20,21).

In stage II, the sandwich flap is isolated from the contiguous skin of the rat abdomen 2 weeks after stage I (see Figure 1B). The sandwich flap is freed from the adjoining abdominal host skin along the original suture line on

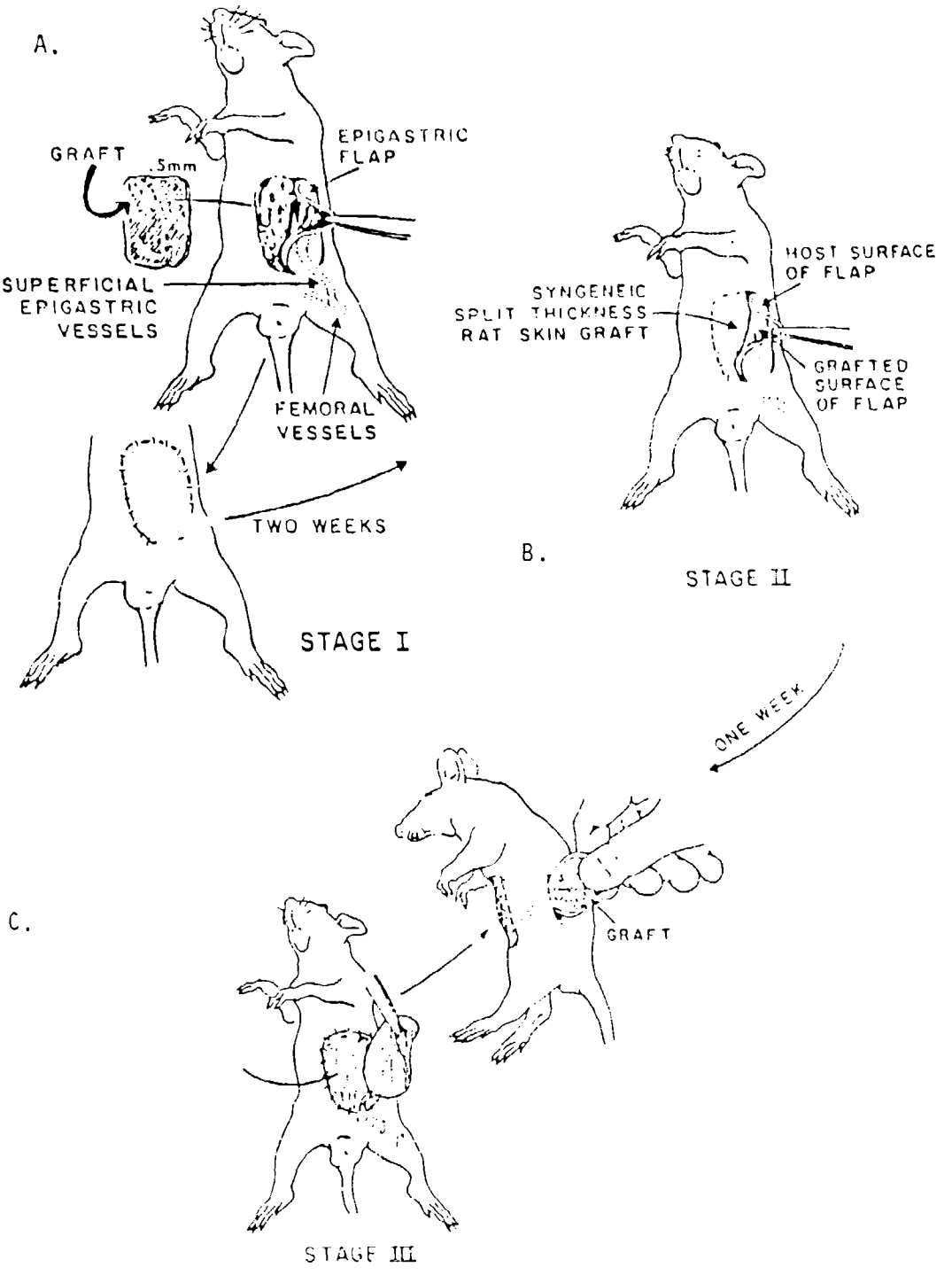

Figure 1. Schematic Diagram of Generation of a Rat-Rat or Human-Rat Skin Flap on the Congenitally Athymic (Nude) Rat. This diagram outlines the procedures for each of the stages utilized in generating the skin sandwich flap on an isolated but accessible vasculature.

three sides with a scissor, leaving the base of the flap intact. Histology (data not presented) reveals that vessels of the subcutaneous surface of the host flap grow into and establish a vascular network with the underlying STSG during this 2-week interval. The skin defect on the host abdominal tissue is fitted with syngeneic split-thickness rat skin (0.5 mm), epidermis side up, and sutured in place with interrupted stitches along the three sides and at the flap base with 5-0 monofilament nylon adhered to a PS-2 needle. Vaseline gauze is applied to both sides of the flap and the rat abdomen to maintain tissue hydration. A bandage dressing of conforming gauze is again applied and left in place for 1 week.

Twenty-one days after the initial surgery (stage I), the flap microcirculation has stabilized, as verified in a series of assessments with the LDV (data not presented). At this time, the vascular pedicle is created (stage III) (see Figure 1C). Isolation of the superficial epigastric artery and vein as the primary source of blood supply and drainage is accomplished with careful dissection utilizing an operating microscope. The vessels medial to the superficial epigastric vasculature, namely the pudendal artery and vein, are sacrificed by ligation and division. This manipulation increases the workable length of the femoral vessels so that the sandwich flap can be transferred to the rat's back. The femoral artery and vein proximal to the origin of the superficial epigastric vessels are ligated individually proximally, together distally, and sacrificed. Bleeding sites are cauterized with a bipolar coagulator. These surgical manipulations maximize blood flow to the flap because all of the blood from the femoral artery which supplies the flap is shunted to the flap. The isolated skin sandwich flap with its attached vascular pedicle is translocated through a subcutaneous tunnel to the rat's back where it is sutured in place. The wound in the inguinal area is closed by mobilizing the surrounding skin into a primary closure. Collateral circulation to the leg is sufficient to maintain the leg's viability and function. Inhibition of chewing on the flap by the rat is accomplished with a restrictive bandage of conforming gauze placed from behind the forelegs to the front of the hind legs in such a way as to leave the flap free and exposed to air. A period of 2 weeks is required to complete the healing following this final surgery before the animal can be used experimentally. A typical skin sandwich flap in place and ready for experimentation is shown in Figure 2.

Accessibility of vasculature supplying skin flap

The reisolation of the superficial epigastric vessels supplying the flap, as they arise from the femoral vessel, is accomplished with a simple incision over the inguinal ligament. The foregoing surgical procedures lead to some hypertrophy of the femoral artery and vein, thus making them more accessible. The opposite groin is exposed in a similar manner. The contralateral femoral vein is used for collection of systemic blood, the former site being used for collection of flap blood. These isolations are performed under the operating microscope at 18X. Heparin (10 IU/100 g body weight in 0.7 ml of saline) is injected subcutaneously and intravenously immediately prior to experimental use to provide a degree of anticoagulation. The venipuncture site to be utilized for the collection of experimental blood samples from the flap is made with a 30-gauge needle approximately 1 cm proximal to the origin of the femoral vein relative to the iliac vein. This 1-cm distance is chosen so that a microclamp can be placed on the femoral vein during blood collection to minimize possible reflux from the systemic circulation. Blood samples (50 ul) are collected in heparinized microhematocrit tubes periodically throughout the experiment via

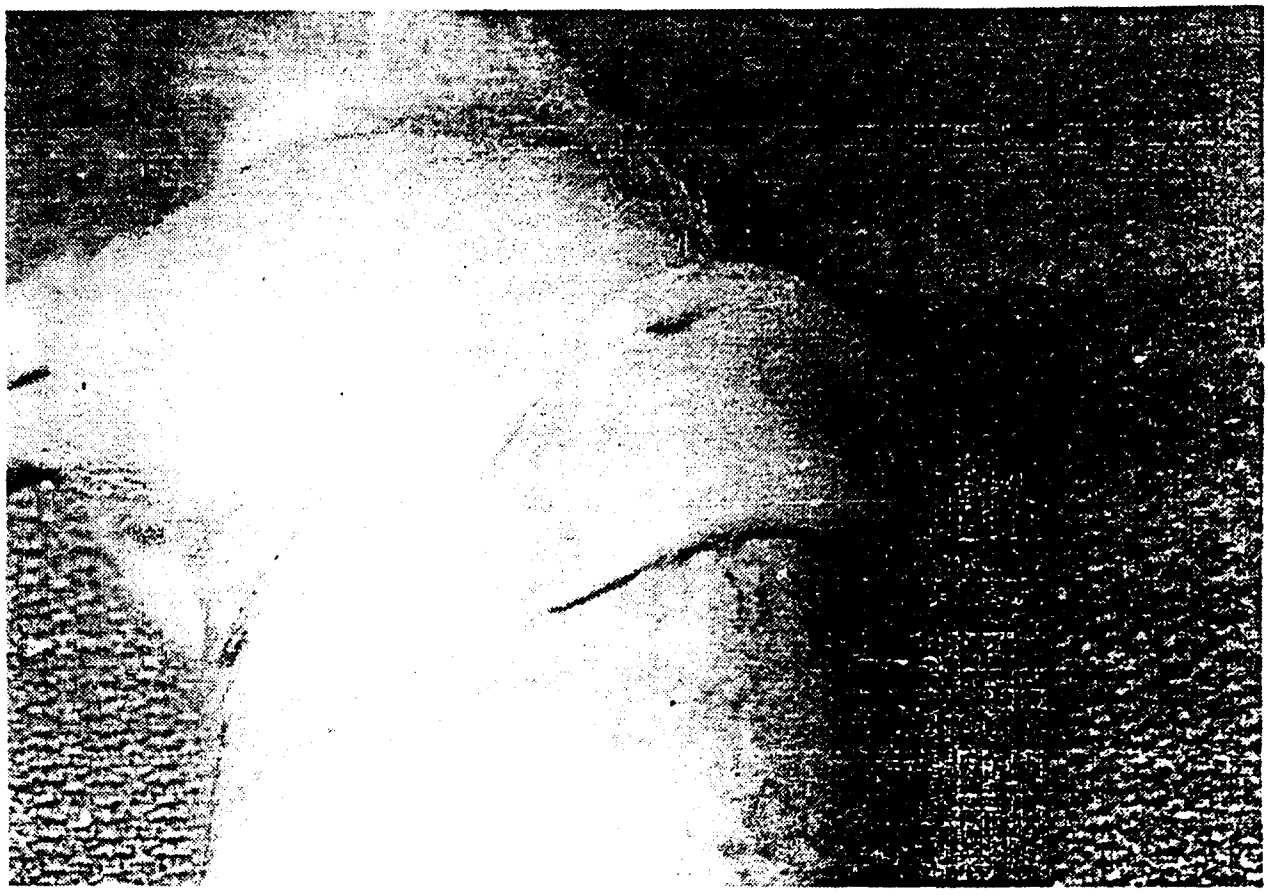

Figure 2. Photograph of a Rat-Rat Skin Sandwich Flap on a Congenitally Athymic Nude Rat. This photograph was taken 3 weeks after stage III. The location of the flap is over the lateral abdomen, immediately anterior to the thigh of the nude rat. The surface depicted in this photograph is that of the rat host.

capillary action when tubes are placed over the venipuncture site. Hemostasis is produced by placing a cotton pledget over the venipuncture site.

Dosage of cyclosporine to maintain human-rat flap

Initially, we reported that subcutaneous injection of 20 mg of cyclosporine on days 1-20 was sufficient to maintain the human skin in a viable state on the nude rat. Persistent graft failure in some animals at later time periods prompted the change to a protocol in which animals receive a 12.5-mg subcutaneous injection of cyclosporine once per week for the duration of the flap life. The animals routinely lose 20-30% of their body weight during this treatment course. The loss of weight coincides with animal sickness which may, in fact, reflect cyclosporine toxicity. Poor animal health may also lead to flap chewing, a significant problem (see Results/Discussion). The cyclosporine treatment protocol was further altered in an attempt to alleviate some of the foregoing problems. Presently, animals receive 20 mg of cyclosporine on day 1 and 12.5 mg subcutaneously every other day until day 21. At day 21, rats are placed on cyclosporine in sterile, pH 2.0, drinking water. The animals receive approximately 11 mg/kg/day when they consume the average daily consumption of water, 30 ml/day. The cyclosporine water bottles are wrapped in aluminum foil due to the drug's photolability. The drinking water is held at a pH of 2.0. Communication with Dr. L. Jacobs, Analytical Chemistry at Sandoz Laboratories, has assured this laboratory that this pH does not lead to degradation or decreased stability of cyclosporine over 2 weeks. The cyclosporine drinking water used in our studies is prepared from the commercially available cyclosporine for intravenous use. Water bottles are changed on a semiweekly basis. Currently, animals lose only 10% of their body weight through day 21; thereafter, they either maintain or demonstrate an increase in body weight.

Percutaneous absorption using the skin sandwich flap

Figure 3 illustrates the model with host skin located on the top and the STSG located on the bottom of the flap. Investigational compounds can be applied to either the host or the graft side of the sandwich flap, either separately or simultaneously, the latter requiring different radiolabels or analytical methods which can detect nonradiolabeled compounds. In the experiments reported herein, the test compound is topically applied in a Teflon well (1.0-cm diameter). The LDV designation at the bottom of Figure 3 refers to laser Doppler velocimeter and illustrates the site of attachment of this instrument.

Percutaneous application of benzoic acid

Benzoic acid has been used by a number of investigators to study percutaneous absorption (1,2,22,23). In our experiments with this agent, nude rats with a healed rat-rat skin sandwich flap are placed on a water heating pad to maintain an internal temperature of 37±0.5°C, the temperature being monitored with a rectal thermometer left in place throughout the entire experiment. Animals are anesthetized as described above and [^{14}C]-benzoic acid (specific activity 56.8 or 19.3 mCi/mM) in phosphate-buffered saline vehicle is deposited onto the skin in the Teflon well attached to the flap skin (400 ul containing ~20 ug of [^{14}C]-benzoic acid). This is immediately covered with Parafilm to minimize evaporation. The Parafilm is periodically lifted and 3-ul aliquots are collected and analyzed for changes in [^{14}C]-benzoic acid concentration in the donor well. Blood samples are collected as described above and analyzed for dpm with liquid scintillation.

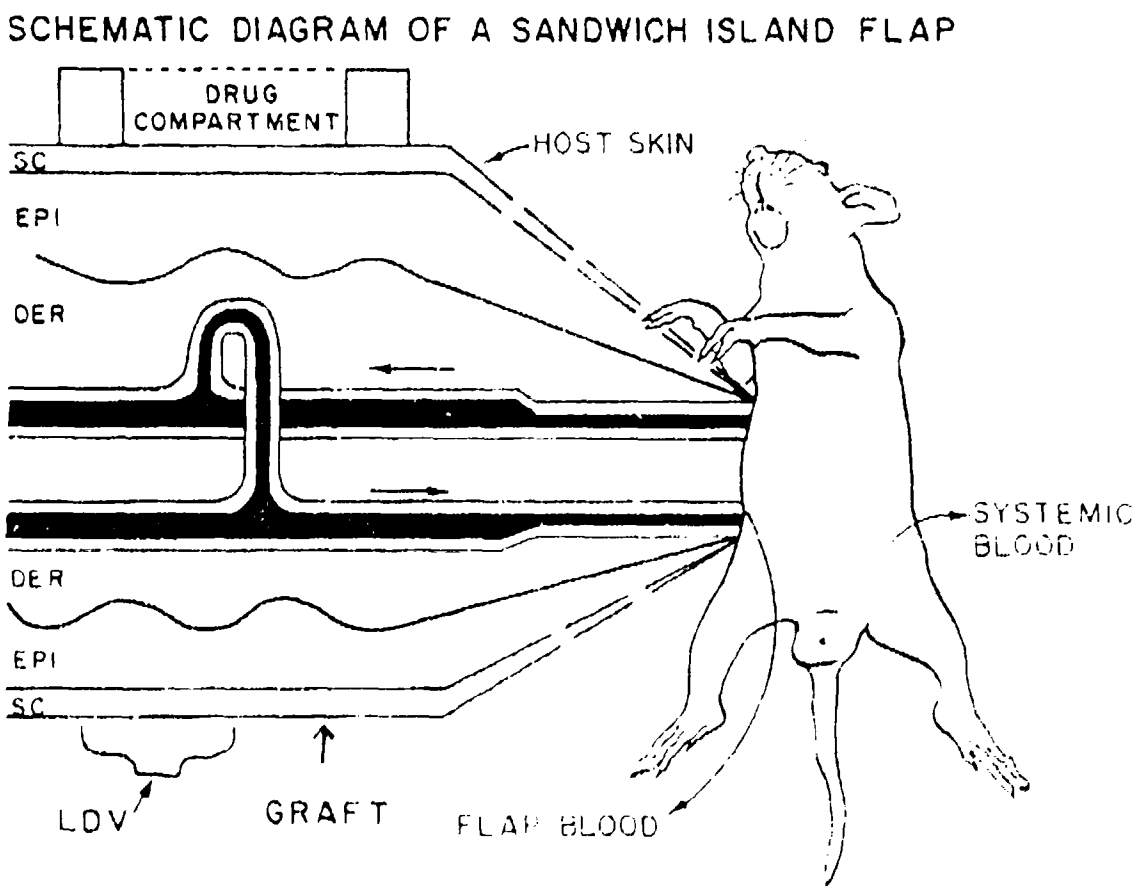

Figure 3. Schematic Diagram of a Sandwich Island Flap. Depicted in this diagram is the compartment where the test drug is applied and the position of the LDV relative to that compartment. Note that both the host and graft surfaces of the flap are served by a common vasculature. In a typical experiment, the systemic blood is drawn from femoral vessels of the groin opposite the origin of the vessels supplying the flap. SC = stratum corneum. EPI = epidermis. DER = dermis.

Percutaneous absorption of caffeine across human, rat host, and rat graft skin

[^{14}C]-Caffeine (specific activity 47.3 mCi/mM) dissolved in ethanol is deposited in a 22-ug dose in a Teflon well. The ethanol is allowed to evaporate and an assessment of percutaneous absorption is made as described for that of benzoic acid (see above).

Assessment of skin distribution of radioactive compound

The flap model system has some limitations for making a complete assessment of the amount absorbed per unit time. These limitations include the length of time the animal can be under anesthesia, the amount of blood that can be taken from an animal at any one time without causing hypovolemia and shock, and the fact that the investigators are unwilling to stay on the job site for more than 10 hr at a time. Therefore, a method was developed to localize the distribution of the test compound in the skin at the end of a 4-hr experiment. The method involves collection of a 2-mm punch biopsy from the test compound-treated area. This 2-mm biopsy is frozen in mounting medium and 50-u sections are collected from one skin surface to the other skin surface (recall that the flap has two epidermal surfaces) with the use of a cryostat. Each of these 50-u sections is digested in 1 M piperidine, mixed with a liquid scintillation cocktail, and the radioactivity determined. From these data, a profile is produced of the distribution of the compound in the treated area of the flap. The total amount of radioactivity remaining in the treated site is calculated based on surface area exposed, roughly 25 times that area contained in a 2-mm punch biopsy. A 2-mm punch biopsy is also collected from another area of skin, typically from the back of the animal, for comparative purposes. Fixing the biopsy in formalin is not an effective way of assessing distribution because compounds that are not covalently linked to skin or to a receptor will freely diffuse from the skin. Assessment of radioactivity of the mounting media, as well as the Parafilm in which the specimen is placed, reveals that the foregoing technique results in minimal leeching of radioactivity from the specimen.

Theoretical considerations and calculations

For the purposes of continuity and maximal relation to the literature, the data are generally presented as a percentage of the applied dose (as calculated from accumulated dpm) or as nanograms in the case of tissue localization experiments (see above). The percent of original dose is calculated as the area under the concentration time curve by the trapezoidal rule. Because blood flow is demonstrated (see Results/Discussion) to be critical to the amount of a compound absorbed over time, data are corrected for blood flow at each collection time period [(ul/min)(ug/ul X min)].

Another useful way to demonstrate the data collected is as flux of the compound into the flap bloodstream. The flux of the study compound into the flap circulation after transdermal absorption across the skin flap is given by the equation: $J_f = (C_f - C_{sys})Q/A$, where C_f is the concentration of the compound in the venous blood exiting the skin sandwich flap, C_{sys} is the concentration of the compound in the recirculating blood, i.e., the systemic blood, entering the flap, Q is the rate of blood flow through the flap, and A is the skin surface area. Area under the curve (AUC) is calculated by the trapezoidal rule as a concentration-time curve (ug/ul X min vs. time). When the concentration-time curve is corrected for blood flow [(ul/min)(ug/ul X min)], the data are plotted as amount of drug (micrograms) vs. time.

Graft rejection studies

The rejection of human split-thickness skin grafts (HSTSG) on nude rats was studied to determine whether this process could be inhibited. These investigations include histologic and immunologic analyses. The HSTSG were orthotopically grafted to the recipient skin site of the lateral thorax, which was surgically excised to the panniculus carnosus. A dermatome (Brown Electro-Dermatome, Model 666, Zimmer Inc., Warsaw, In) was used to prepare the HSTSG (0.4 mm in thickness) from remnants of fresh normal human skin, voluntarily donated by patients undergoing elective plastic surgery. These were trimmed to the desired size, 1-4 cm in diameter, transplanted to the recipient site, and sutured in place with 5-0 silk. The grafts were covered with a Vaseline-impregnated gauze and covered by wrapping the thoracic cage with several layers of surgical tape. The dressings and sutures were removed on day 10. The grafts were inspected daily for signs of rejection. Rats that rejected their primary grafts were selected for regrafting 4-6 weeks after rejection of the primary graft.

Histology and immunofluorescence studies

Biopsies were taken from HSTSG before, during, and after rejection. Histologic sections from these biopsies were stained with hematoxylin and eosin. Cryostat sections (6 uM) of skin were prepared for indirect and direct immunofluorescent assessments of immunoreactants in the skin and serum. The reagents used were fluorescein-labeled goat anti-rat IgG, IgA, IgM, and anti-rat complement (Cappel Laboratories, Cocherville, PA).

Treatment protocols

To prevent graft rejection, treatment protocols were directed at the animal before and after engraftment or at the graft before engraftment. The size of the treatment groups varied and was never more than eight nor less than two.

Treatment of skin in vitro prior to transplantation

Blocking the effect of class II histocompatibility antigens: Reports of prolongation of skin allografts by incubating the graft in antisera specific for these antigens (24) prompted the following experiments. Class II molecules in the donor tissue were blocked by incubating HSTSG with anti-human HLA-DR (Becton-Dickinson Monoclonal Center, Inc., Mountain View, CA) at a concentration of 1:20 in buffered saline for 48 hr. Assessment of antibody binding was monitored by secondstep fluorescent technology, which demonstrates the well-established binding of anti-HLA-DR to Langerhans cells and undefined cells of the dermis.

Incubation in vitro: Depletion of cellular as well as soluble mediators of the skin, which may facilitate graft rejection, was accomplished by culturing HSTSG with RPMI-1640 supplemented with 10% fetal calf sera (FCS) for 7 days at 37°C (25).

X-irradiation: HSTSG were x-irradiated with 1000 r (580 rads/min), using gamma irradiation from cesium source (Biomedic Inc., Parsippany, NJ), to decrease the likelihood of humoral cellular components within the skin grafts initiating the rejection process.

Treatment of animals prior to or after engraftment with HSTSG

Whole body x-irradiation: Nude rats were treated with a single whole body dose of x-irradiation (500 r) 1 hr prior to skin transplantation, under the direction of J. A. Sorenson, Ph.D., Director of Medical Physics, Experimental Radiation Oncology, University of Utah School of Medicine, Salt Lake City, UT. These animals received neomycin/polymixin cocktail in their drinking water for 1 month; each 250 ml of water contained 25 mg of neomycin and 2.5 mg of polymixin (26).

Anti-lymphocyte serum (ALS): Prior to utilization of the ALS, kindly supplied by C.W. DeWitt, Ph.D., Department of Pathology, University of Utah School of Medicine, an in vitro analysis with dilute agent demonstrated cytotoxicity against nude rat lymphocytes. Following engraftment, rats were injected intravenously for 4 consecutive days and then weekly for 7 weeks with 0.5 cc of undiluted ALS. This treatment did not produce lymphocytopenia but did produce significant weight loss.

Anti-asialo GM 1: Nude rats exhibit increased natural killer (NK) cell activity (11). The antiserum to the molecular species asialo GM inhibits NK activity (27). Anti-asialo GM 1, rabbit (Wako Chemicals USA, Inc., Dallas, TX), about 10 mg/ml, plus complement, eliminated NK activity inherent to splenocytes from nude rats when incubated in vitro and assessed for activity in a standard NK assay (27). Therefore, grafted rats were given an intravenous injection of 0.5 ml of anti-asialo GM 1 (diluted 1:6 or 1:12, as supplied, with phosphate-buffered saline (PBS)) 2 days before grafting, 4 and 8 days after engraftment, and every 7th day thereafter to inhibit graft rejection.

Cyclosporine: Cyclosporine (CS), 50 mg/ml (Sandoz, East Hanover, NJ), was injected subcutaneously, 25 mg/kg/day for 21 consecutive days. Weight loss was routine; hence, the dosage was lowered as the therapy continued. A group of animals that rejected HSTSG was regrafted 4-6 weeks after the first rejection; one half of this group received the foregoing dose of CS for 21 days and the other half was left untreated. Another group that rejected the primary graft was regrafted and treated with CS for 60 days. Data collected were analyzed for days of successful engraftment.

Bordetella pertussis toxin: Bordetella pertussis toxin (BPT) is known to prolong grafts across major histocompatibility (MHC) barriers (28). Rats received either purified BPT (2000 ng/rat), kindly supplied to us by G. Spangrude, Ph.D., Department of Pathology, University of Utah School of Medicine, or BPT and CS. All animals in this series received a single intravenous dose of purified BPT (2000 ng) 2 days prior to skin grafting. A single group also received Cs (25 mg/kg/day) for 21 days in addition to BPT. The final group received a second dose of BPT 1 week after grafting. Blood smears from these groups and the nontreated controls were analyzed for BPT-induced lymphocytosis.

Orthotopic transplantation of split-thickness scalp grafts

These studies were designed to determine whether: 1) human scalp skin could be grafted onto the nude rat, 2) STSG thickness influenced the density of subsequent hair growth, and 3) CS therapy could alter hair growth. The human skin utilized in these experiments consisted of remnants of scalp skin removed during elective "face-lift" surgery. These remnants were shaved initially with

a safety razor to remove the existing hair and then trimmed to the appropriate thickness (0.4 mm or 1.0 mm) with a dermatome. Rats were anesthetized with ketamine hydrochloride, 1 mg/10 g body weight (Ketalar, Parke-Davis, Morris Plains, NJ), before engraftment. The skin overlying the recipient site, the lateral thorax, was surgically excised to the depth of the panniculus carnosus to a size appropriate for the HSTSG of scalp skin (HSTSG-SS). Bleeding was controlled with pressure. The HSTSG-SS was cut to the desired size (1-4 cm in diameter) with a sterile cork borer, placed on the recipient site, and sutured in place with 5-0 silk sutures. The graft sites were covered with a Vaseline-impregnated gauze and further secured by wrapping the thoracic cage with several layers of surgical tape. The dressings and sutures were removed on day 10; thereafter, the grafts were inspected daily for signs of rejection.

CS (50 mg/ml, intravenous solution, Sandoz Laboratories, East Hanover, NJ) was injected subcutaneously, 25 ug/kg/day, to 10 rats grafted with 0.4 mm thick HSTSG-SS and to five rats grafted with 1 mm thick HSTSG-SS. Four rats were grafted with 0.4 mm thick HSTSG-SS, treated with: 1) intravenous injections of 0.5 cc of undiluted ALS (n=2) on 4 consecutive days and then once per week for 7 weeks, 2) azathioprine (Imuran, Burroughs Wellcome Co., Research Triangle Park, NC), 5 mg/kg/day intraperitoneally (n=1), or 3) intraperitoneal injections of azathioprine (5 mg/kg/day) and hydrocortisone succinate, 0.5 cc (Solu-Cortef, Upjohn Co., Kalamazoo, MI), on a daily basis for 5 weeks. Discontinuation of ALS resulted in graft rejection within 9 days. Rejection of HSTSG-SS occurred in 29±9 days without immunosuppressive therapy.

Biopsies were collected from the HSTSG-SS before and at intervals following engraftment. Fixation of tissue in 10% neutral buffered formaldehyde solution was utilized for routine histologic sections. Biopsies were sectioned horizontally or vertically, stained with hematoxylin and eosin, and examined under light microscopy.

Hair growth was also assessed in terms of the number of hairs per graft and hair length. These measurements were evaluated under 5X magnification. Hair shaft diameter was measured on transverse sections with the use of a calibrated ocular micrometer (29).

Results/Discussion

Incidence of rejection

Grafting STSG onto nude rats (n=10) from allogeneic rats revealed no evidence of graft rejection in this laboratory or others (11) over a 4-month observation period. However, 89% (50/56) of nude rats grafted with HSTSG slowly rejected their grafts between the 3rd and 6th week of engraftment. Natural killer cell activity is low in the early life of nude rats (11). Therefore, 16 animals were grafted at 3 weeks of age with HSTSG. All of the rats rejected the grafts during the same time period observed for the older nude rats in the previous study (data not shown).

When these animals were challenged with a second set of grafts, rejection was more rapid and complete (7-21 days). The six rats that accepted HSTSG initially accepted a second HSTSG. Implanting human skin in the subcutaneous tissue at birth in animals of two litters, one-half nude and one-half heterozygous for nude (n=8), revealed no evidence of neonatal tolerance, since

rejection of a second HSTSG occurred when regrafted at 3-5 weeks of age in both genetic types. There was no difference in rejection rate between sexes. Female rats rejected grafts after a mean of 26 days, while males rejected grafts after a mean of 29 days. The immunologically normal littermates, heterozygous for the athymic state (nude) (11), rejected first set HSTSG more rapidly than nudes, 16 days vs. 29 days for nudes.

A comparative analysis of anatomic sites for the graft (back, thoracic cage, and burial in the subcutaneous space), as well as the diameter of the skin grafts (4 mm to 2.0 cm, n=3 for each group), revealed that rejection was not dependent on either factor. It was curious to note that of the rats that accepted HSTSG, there were littermates who received the same skin, at the same time, who eventually rejected the grafts.

Definition of the immune status of nude rats that reject HSTSG

The unexpected rejection of xenogeneic grafts of human skin, coupled with an awareness that similarities between the skin and the thymus (30) might have led to a situation in which an athymic animal is, at least in part, thymically reconstituted, led to the review of components of T-cell-mediated function in rats rejecting HSTSG. Autopsies and histologic exam of athymic rudiments and lymph nodes of three nongrafted nudes and three grafted nude rats, within 7 days of rejection of HSTSG, revealed no increase in the thymic dependent components of the draining lymph nodes and no evidence of the generation of thymic tissue when compared with their normal heterozygous littermates.

Splenocytes and lymphocytes from spleens and lymph nodes (n=3 per group) were challenged with 0-25 ug of the T-cell mitogen, concanavalin A/$1x10^6$ cells/ml. Analysis of the blast transformation induced by this mitogen was performed by measuring [^{3}H]-thymidine incorporation at 48 hr. Data revealed a peak response at 2.5 ug in the heterozygous controls, with no response at any dose by either the grafted or the nongrafted nude rats. A mixed lymphocyte response was assessed using the foregoing cell sources challenged with Ficoll-hypaque-isolated mitomycin-treated human peripheral blood mononuclear cells at a response to stimulator ratio of 2:1 in a standard assay of this type (31). In the heterozygous nudes, the mean stimulation index at day 4 was 16.3 for splenocytes and 13.6 for lymphocytes. The stimulation index for the ungrafted nude rats never exceeded 1.5. This contrasts with a mean stimulation index of 3.4 (range of the index = 2.5-3.9) for lymphocytes from the four grafted nudes. Portions of these cultures were analyzed for lytic activity against concanavalin A blast-transformed peripheral blood mononuclear cells from the same donor and radiolabeled with chromium (^{51}Cr) at day 4 (31). Data demonstrate that lymphocytes, but not splenocytes, from grafted nude rats will lyse these blast-transformed peripheral blood mononuclear cells at an effector to target ratio of 25:1. In this analysis, grafted nude rats produced 34% lysis, while the nongrafted nude and the heterozygous controls had 4% and 7% lysis, respectively.

Natural killer cell activity in splenocytes and lymphocytes of the peripheral lymph nodes was also investigated, again using a standard assay (27). Nongrafted nude rats had up to 6 times the lytic activity of heterozygous littermates. Lytic activity increased to 12-fold with splenocytes from nudes that had recently rejected HSTSG. Similar responses in lytic activity were noted with lymphocytes.

To capsulize, no histologic evidence of thymic reconstitution or evidence of an enhanced response to a T-cell mitogen was detected in the nude rats grafted with HSTSG. There is, however, evidence of a mixed lymphocyte reaction with lymph node lymphocytes which correlated with a concomitant increase in lysis of target cells, and an increased natural killer cell activity in spleens and lymph nodes following engraftment and rejection of HSTSG.

Histopathology of the rejection of HSTSG by the nude rat

The expense of the nude rat, the variable and long time period to rejection, and the difficulty of precisely determining the onset of graft rejection have precluded an analytic histopathologic assessment of the rejection process, as a function of time. The time from the earliest visual change indicating graft rejection to ulceration ranged from 6-9 days.

Transplanted HSTSG to the nude rat which was undergoing rejection had a variable hyperkeratotic and acanthotic epidermis when histologic sections were examined microscopically. Along the basal cell layer of the epidermis, intermittent areas of microvesiculation were noted. These appeared to be secondary to a coalescence of vacuoles within the basal cells. Associated with this change was the presence of individually necrotic, hypereosinophilic keratinocytes (colloid or Civatte bodies). Frequently, these colloid bodies were in approximation to mononuclear inflammatory cells in a pattern described as "satellite cell necrosis." A moderately intense lymphoid infiltrate was present in the lower epidermis and within the somewhat edematous papillary dermis. The dermal component of the inflammatory infiltrate was both interstitial and perivascular. Macrophages filled with melanin were frequently found within the immediate subepidermal zone of the dermis, usually in association with the microvesiculation of the basal cell layer. Extravasation of red blood cells in the papillary dermis was a variable feature. Later in the course of this host vs. graft reaction, the cytologic degeneration of keratinocytes progressed to the point of confluent coagulation necrosis of the epidermis and, finally, to erosion and ulceration.

Direct and indirect immunofluorescent analysis

The results of direct immunofluorescence of various structures within the HSTSG from nude rats are shown in Table 1. Table 2 summarizes the results of indirect immunofluorescence using normal and successfully grafted human skin (n=2) (HSTSG without signs of rejection at 90 days following engraftment) as substrates for sera from two groups of rats: one group that had been grafted with and rejected HSTSG for the first time, and another group that had never been grafted. An analysis of these sera, diluted 1:20, revealed that all grafted rats undergoing rejection had circulating antibodies which bound to various structures in skin. This contrasts with the relative absence of these immunoreactants in the serum of nongrafted rats.

The specific binding pattern of these antibodies to a nongrafted human skin substrate differed from that seen with nonrejected HSTSG. Binding of IgG in the nongrafted HSTSG was noted to the stratum corneum in 12/28 (42.8%), to BMZ in 10/28 (35.9%), to major hair follicles in 7/28 (25%), and to blood vessels in 6/28 (21.4%). The major binding of IgM was in the stratum corneum, 16/28 (57.1%), with a nuclear pattern in the epidermis in 11/28 animals (39.2%). These results are in contrast with the results observed when these sera were tested with successfully grafted HSTSG as the substrate. All (100%) of the sera from rats undergoing rejection had circulating IgG to the BMZ of

Table 1. Direct Immunofluorescence of Grafted Human Skin[1]

	IgG	IgM
Basement membrane	3/4[2]	0/4
Blood vessels	0/4	0/4
Stratum corneum	1/4	0/4
Cytoplasmic pattern[3]	0/4	0/4
Nuclear pattern[3]	1/4	1/4

[1]Analysis of human skin grafts in early stages of rejection.
[2]Values = number positive per number tested.
[3]Binding of rabbit anti-rat IgG or IgM to cytoplasmic components or nucleus.

Table 2. Indirect Immunofluorescence using Different Substrates

	Normal Human Skin[1]				Grafted Human Skin[2]	
	Serum: Normal[3]		Serum: Rejected[4]		Serum: Rejected[5]	
	IgG[6]	IgM	IgG	IgM	IgG	IgM
Basement membrane	1/8	0/8	10/23	3/28	6/6	1/6
Blood vessels	0/8	0/8	6/26	4/28	4/6	0/6
Stratum corneum	2/8	1/8	12/28	16/28	0/6	2/6
Cytoplasmic pattern	1/8	0/8	5/28	7/28	0/6	0/6
Nuclear pattern	0/8	0/8	4/28	11/28	0/6	2/6
Hair follicles	0/8	0/8	7/28	4/28	0/6	0/6

[1]Cryostat sections of remnants of STSG used for grafting.
[2]Cryostat sections of human STSG removed from a nude rat without evidence of rejection.
[3]Serum from eight nongrafted nude rats analyzed for binding to cryostat sections.
[4]Serum from 28 nude rats having rejected human STSG.
[5]Serum from six nude rats having rejected human STSG.
[6]Class of immunoglobulin tested with appropriate secondary reagents.

this substrate, while 67% had circulating IgG to blood vessels. Binding of IgM in the sera of graft-rejected rats to these skin components in this substrate was unusual, with no binding to blood vessels and binding to the BMZ in only 16% of the sera. In the sera from the nongrafted rats, 12.5% had antibodies which bound to the BMZ, and none had antibodies which bound to blood vessels.

Additional indirect immunofluorescent analyses were performed on sera collected at weekly intervals from seven rats following rejection of HSTSG. The circulating antibodies to the BMZ and blood vessels, which were detected in the rejection period, disappeared 5-7 weeks after the HSTSG was rejected. These analyses were also performed on sera from four grafted rats being treated with cyclosporine (see below). No circulating antibodies to antigens in the successfully grafted HSTSG substrate were detected in this group of rats.

Finally, the binding pattern with HSTSG from nude mice as the substrate was analyzed with the aforementioned sera. The binding pattern was similar to that observed with never-grafted skin. These data suggest that neoantigens either appear in the BMZ and blood vessels or are expressed in these structures in higher density in skin that remains successfully engrafted on the nude rat.

The foregoing observations suggest that antibodies play a major role in the rejection of the HSTSG. The intravenous transfer of sera (0.5 ml X 5 days) from rats that had just rejected HSTSG into two rats that had HSTSG in place without treatment for more than 90 days resulted in rejection of the HSTSG. Rejection was complete within 14 days of initiating this treatment. Immunofluorescent analysis of skin biopsies from these animals at day 7 for immunoreactants showed deposition of IgG at the BMZ to be the prominent feature.

Treatments to prevent rejection of HSTSG by nude rats

Treatment protocols were developed for the HSTSG before grafting or for the recipient rat before and/or after engraftment to circumvent the rejection process. As demonstrated in Table 3, incubation in vitro for 7 days at 4°C and 1000 r of x-irradiation did not prolong engraftment. These grafts were still viable and survived for greater than 14 days. Extensive experience in this laboratory has demonstrated that nonviable skin will not vascularize and hence will be completely necrotic by day 14. Stimulation of allogeneic T-cell proliferation in vitro has been shown to correlate with the presence of Ia antigens on the stimulating cells (32). Antisera to Ia will block this reaction (33). To establish whether Ia had a role in the HSTSG rejection by nude rats, grafts were either incubated with a monoclonal antibody to the Ia equivalent of man, anti HLA-DR, or with media alone. This treatment also failed to prolong skin survival.

A single whole body dose of x-irradiation (500 r) 1 hr prior to skin grafting resulted in graft survival for 38 days, an insignificant increase. Intravenous administration of BPT and anti-asialo GM also did not prolong engraftment in a significant way (mean survival=44 and 34 days, respectively). Three rats died during treatment with anti-asialo GM in the 1st week of treatment. Therefore, two additional animals were treated with only one half of the original dose. The graft survival time shown in Table 3 for this group is a mean of these two animals; the deaths and the failure to prolong engraftment suggested that further experiments with this agent were not warranted.

Table 3. Protocol Trials to Prolong Human STSG on Nude Rats

	Control	Ia	Media	X-ray	ALS	ASGM	BP	CS
Graft survival[1]	28 (x̄+SEM) 8.55	20	20	38	55	34	44	>90[2]
Rejected/grafted	10/10	2/2	2/2	2/2	2/2	2/2	2/2	0/5[3]

[1]Measured in days.
[2]2/5 rejected grafts; mean time to rejection was 125 days.
[3]3/5 showed no rejection at the time of death, 134.3±15.37 days.

Assessment of blood flow to the flap

Blood flow is acknowledged to be a critical factor in percutaneous absorption, but to date has been unquantified (1). Hence, an in-depth assessment has been made of blood flow to and from the microcirculation in the flap.

Collateral circulation: Prevention of tissue anoxia has been demonstrated to increase the potential for development of collateral circulation through the flap (17). Therefore, procedures utilized to generate the skin flap were designed to maintain a milieu in which tissue anoxia is prevented at all stages. Two experiments were performed to define collateral circulation. Injection of India ink into the femoral artery supplying the flap resulted in the appearance of the ink throughout the flap, with very little in the surrounding skin (Figure 4). The presence of ink in the STSG and host sides of the flap has been confirmed histologically (see Figure 5). India ink is found in all arterioles, capillaries, and venules, with minimal evidence of ink in the vessels of the skin to which the flap is attached.

Further assessment of collateral circulation was made with a dermofluorometer (DF) following an intravenous injection of fluorescein (1.6 mg). The influx and clearance of fluorescein were monitored with a surface fluorometer over time. Occluding either the artery or the vein permits assessment of collateral blood supply. Experiments demonstrated that occlusion of the vein draining the flap decreased the clearance of fluorescein. Relief of occlusion led to prompt clearance. Likewise, occlusion of the artery resulted in the failure of fluorescein to appear in the flap. These data demonstrated that the collateral circulation to the flap was minimal, estimated to be less than 10% of the total blood supply to the flap via the superficial epigastric system.

Flap blood flow postsurgery: Blood flow was assessed by recording the LDV value at various skin sites. Increased blood flow correlates with incfreased LDV values. Changes in blood flow resulting from surgery in stages II (raising the flap free from the rat's abdomen) and III (isolating the flap vasculature with translocation to the rat dorsum) were analyzed with the LDV (12-14). Data gathered from three areas (proximal, media, and distal) on the host side of a sandwich flap over a period of 4 weeks are shown in Figure 6. Although transient increases in blood flow were seen in all three areas up to 1 week following stage II surgery, blood flow returned to original levels and did not change significantly ($p>0.05$) following stage III surgery. A comparison of blood flow in the proximal or distal areas of the flap with the medial area revealed no significant differences on a given day.

Figure 4. Photograph of a Rat-Rat Skin Sandwich Flap Injected Intra-arterially with India Ink. Note the sharp line of demarcation between the flap and the animal. This sharp demarcation illustrates the lack of collateral blood supply to the flap.

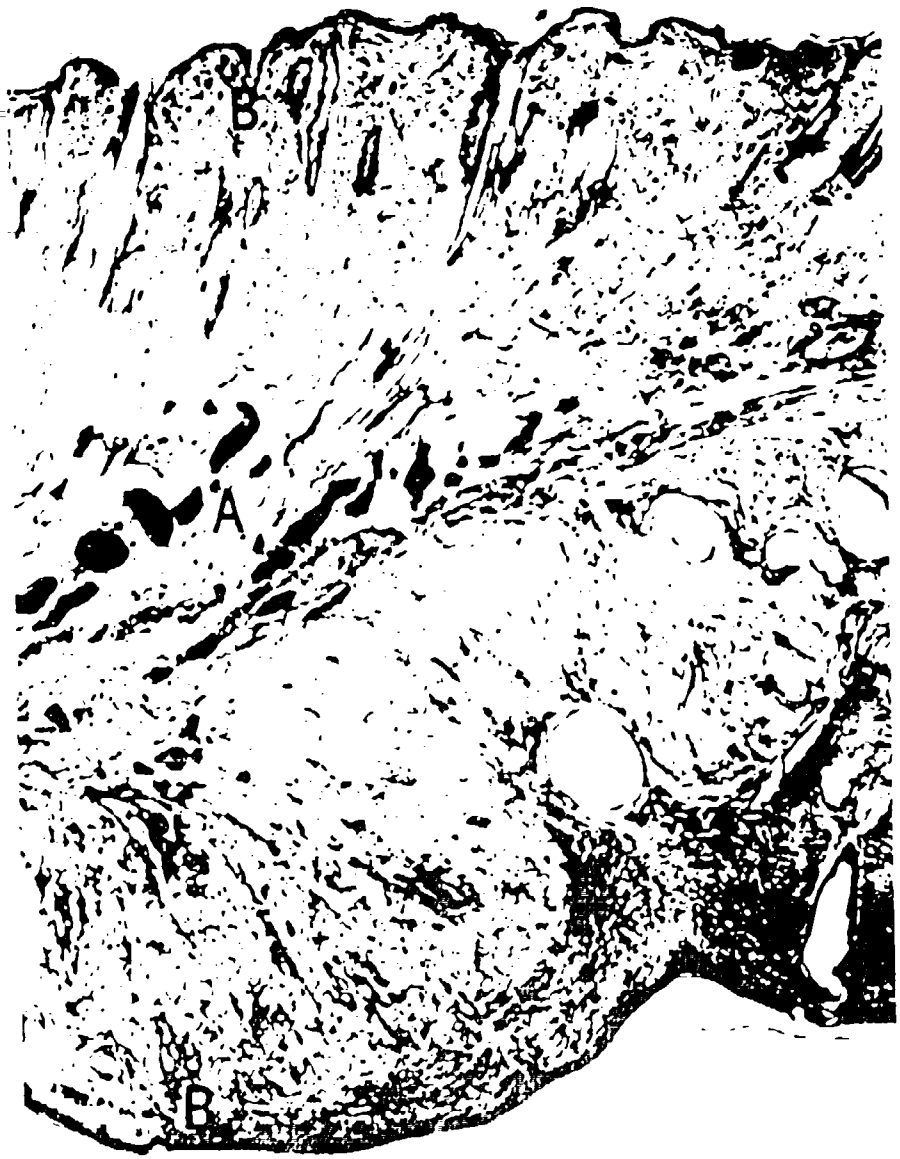

Figure 5. Photomicrograph of a Rat Skin Sandwich Flap after Injection with India Ink and Sacrifice. A represents the relatively large subdermal vasculature. B represents capillaries near the epidermis. Both types of vessels are filled with India ink and thus are seen as black structures.

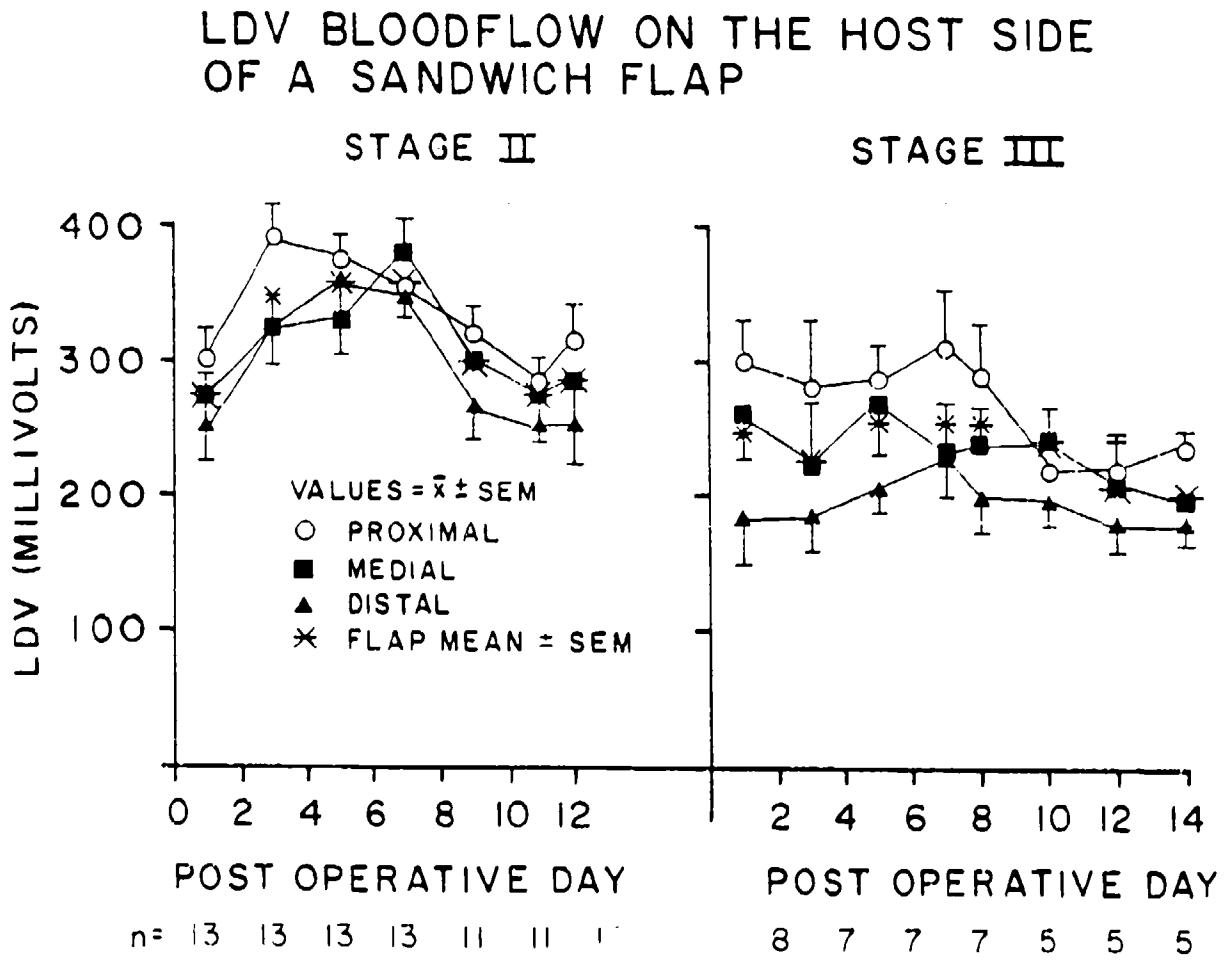

Figure 6. LDV Blood Flow on the Host Side of a Sandwich Flap. Note blood flow in the flap in three areas following stage II and III surgeries as measured by laser Doppler velocimetry on the rat host.

Blood flow volume to the flap: Blood flow volume to the rat-human flap was quantitated using an electromagnetic blood flow meter (EBFM). The C-type transducer attached to the flow meter is affixed to the isolated femoral artery or vein immediately distal to the origin of the superficial epigastric vessels that drain and supply the flap. Measurement of blood flow to eight mature flaps (3 weeks or older), which have about 8 cm^2 skin on each side, demonstrated that the flap blood supply ranged from 1.5 to 2.0 ml/min. Similar values were obtained on the vein draining the flap. Zero values for calibration were made by clamping the artery approximately 1.5 cm proximal to the flow transducer.

Correlation of dermofluorometer, laser Doppler velocimeter, and electromagnetic blood flow meter: Presently, the LDV is used to noninvasively monitor flap blood flow throughout an experiment. The DF noninvasively monitors blood flow utilizing another technique in which the appearance and clearance of fluorescein dye are monitored in the flap. This technology directly correlates with capillary blood flow and renal clearance. To determine the effect of altered microcirculation on absorption, a method that does not itself alter absorption was needed to alter the microcirculation in the skin sandwich flap. This was accomplished by delivering phenylephrine, ar alpha agonist which induces local vasoconstriction, to a test site by iontophoresis. The data are plotted as the relative index of LDV against DF (Figure 7). The relative index was calculated by determining the experimental DF value in the vasoconstricted area with that of control area. The percent relative index for the LDV was calculated in a similar manner. This correlative analysis demonstrated a high degree of correlation, r=0.95, which confirmed that the LDV was, in fact, measuring blood flow. The LDV, a noninvasive and convenient tool for monitoring flap blood flow, does not measure actual volume per time of blood flow. Therefore, the LDV was compared and correlated with the EBFM, an invasive instrument utilizing a "C" probe directly attached to the artery supplying the flap. This instrument directly measures blood flow in milliliters per minute. A correlation experiment between the two instruments was performed utilizing a nude rat (W1) weighing 179 g on cyclosporine drinking water with a healthy rat-human skin sandwich flap. Blood flow was manipulated by altering the temperature of the animal and by mechanical pressure proximal to the origin of the superficial epigastric artery. Using these manipulations, whenever the EBFM read 0, 1, 2, or 3 ml/min, the corresponding LDV value was recorded. The LDV readings were: 36 mv at 0 ml/min, 124 mv at 1 ml/min, 261 mv at 2 ml/min, and 440 mv at 3 ml/min (Figure 8). The correlation between the two instruments was r=0.993. The value of 55 mv, without evidence of blood flow, is a value routinely observed, e.g., attaching the LDV to nonviable human skin prior to grafting results in LDV values ranging from 35 to 60 mv.

A similar assessment (data not presented) was performed at the end of stage II. In that assessment, the slope of the correlation is not as steep as the former, 13.5 mv/ml/min vs. 37.7 mv/ml/min, respectively. This analysis confirmed our contention that the microvascular surgery at stage III, surgery to ensure a sole source of blood supply to and from the flap, does indeed reduce the collateral circulation to the flap by approximately 25 mv.

Blood flow to the host and graft sides of the flap: An assessment of blood flow to the nongrafted and grafted sides of the flap was performed utilizing the LDV at a variety of flap ages (Figure 9). In these numerous

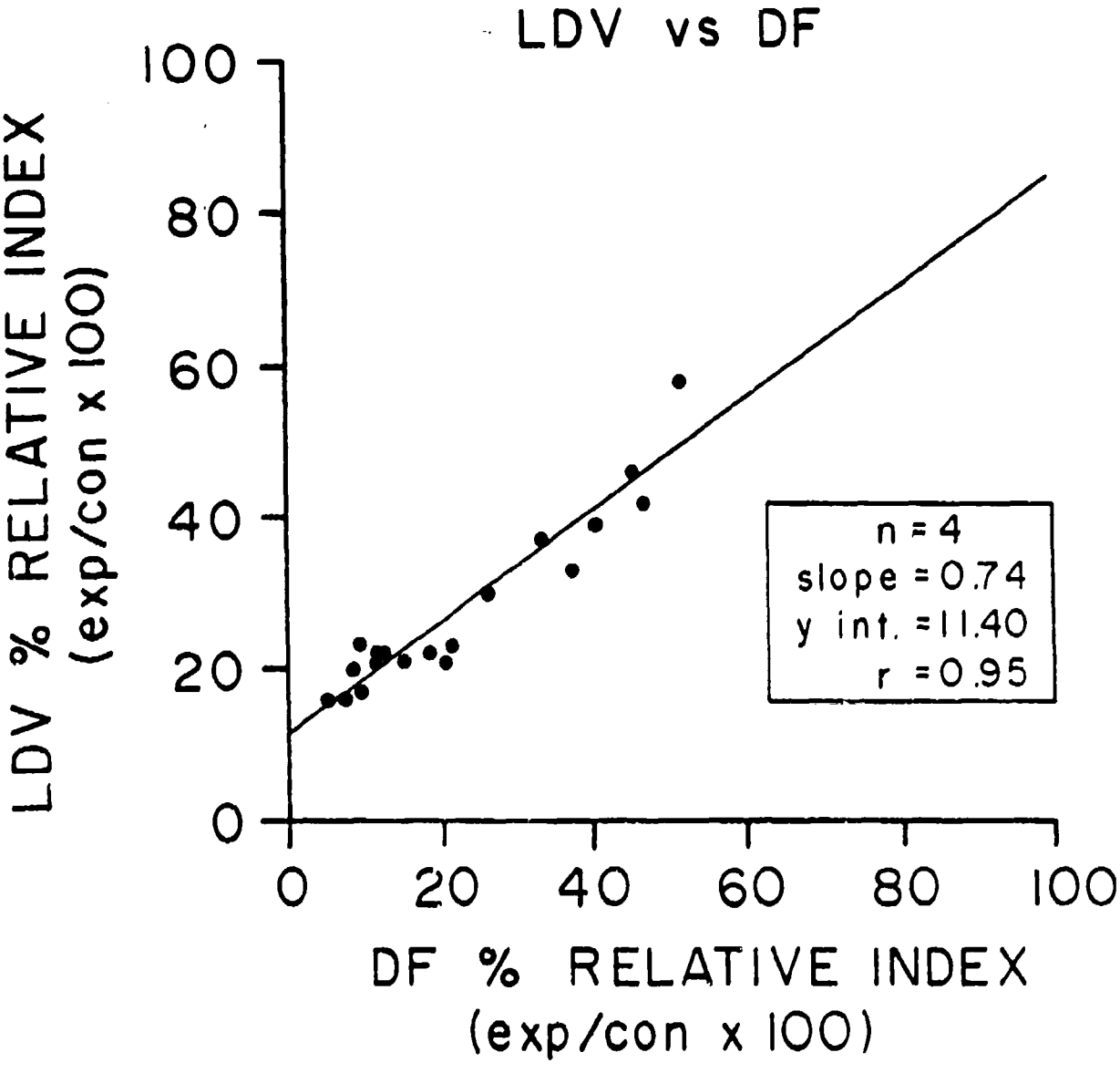

Figure 7. Correlation of LDV Values with Clearance of Fluorescein from the Skin as Measured with the Dermal Fluorometer (DF). Vasoconstriction was accomplished with delivery of phenylephrine via iontophoresis, and the relative index was calculated by comparing instrument reading values in the vasoconstricted area with those immediately adjacent to the vasoconstricted area. A highly significant correlation is present.

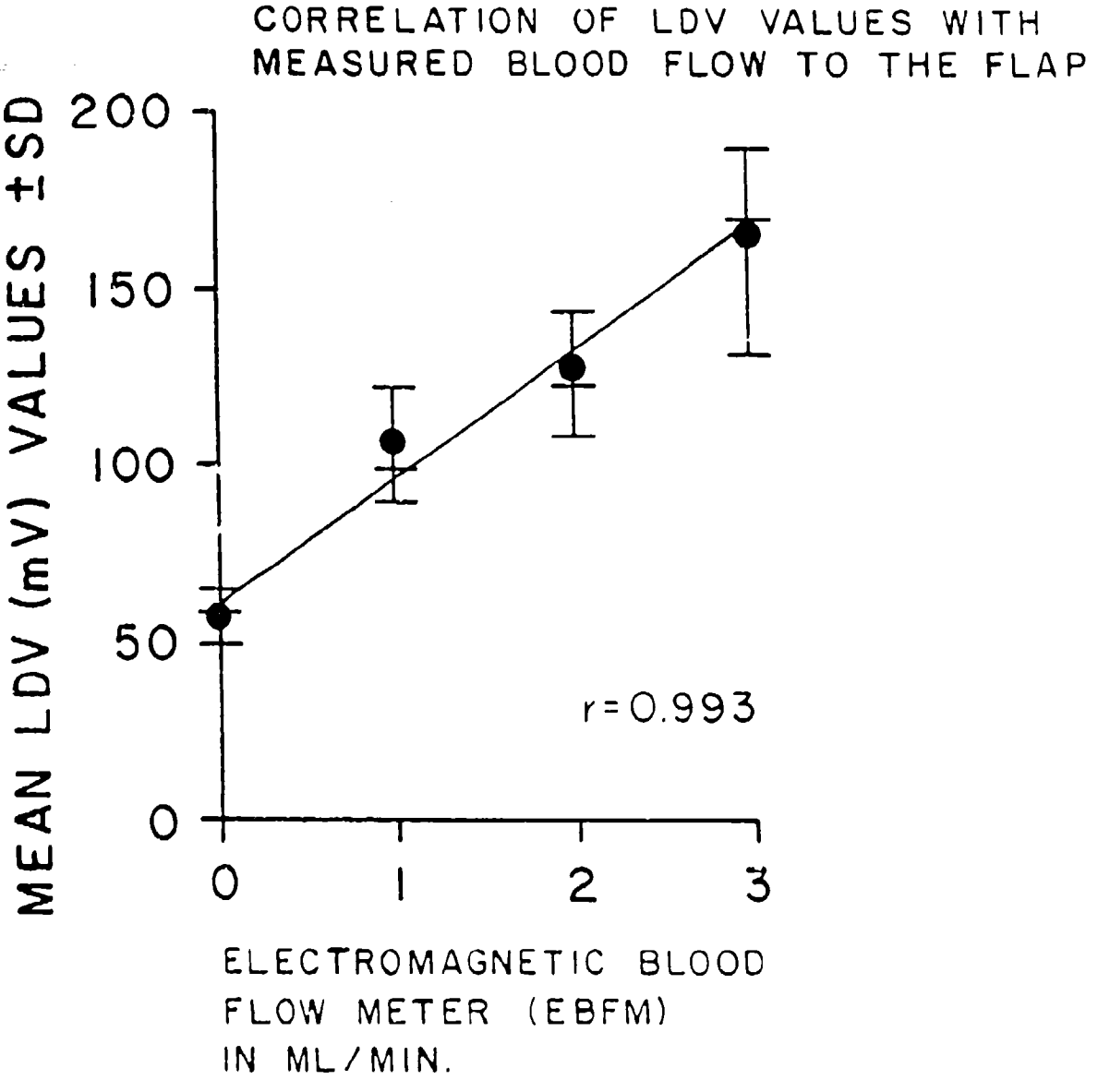

Figure 8. Correlation of LDV Values with Measured Blood Flow to the Flap. In this experiment, blood flow of the artery supplying the flap was measured with an electromagnetic blood flow meter in milliliters per minute. When these values were 0, 1, 2, or 3, LDV values were recorded, n=4. These numbers were plotted, revealing a highly significant correlation between LDV values and actual blood flow.

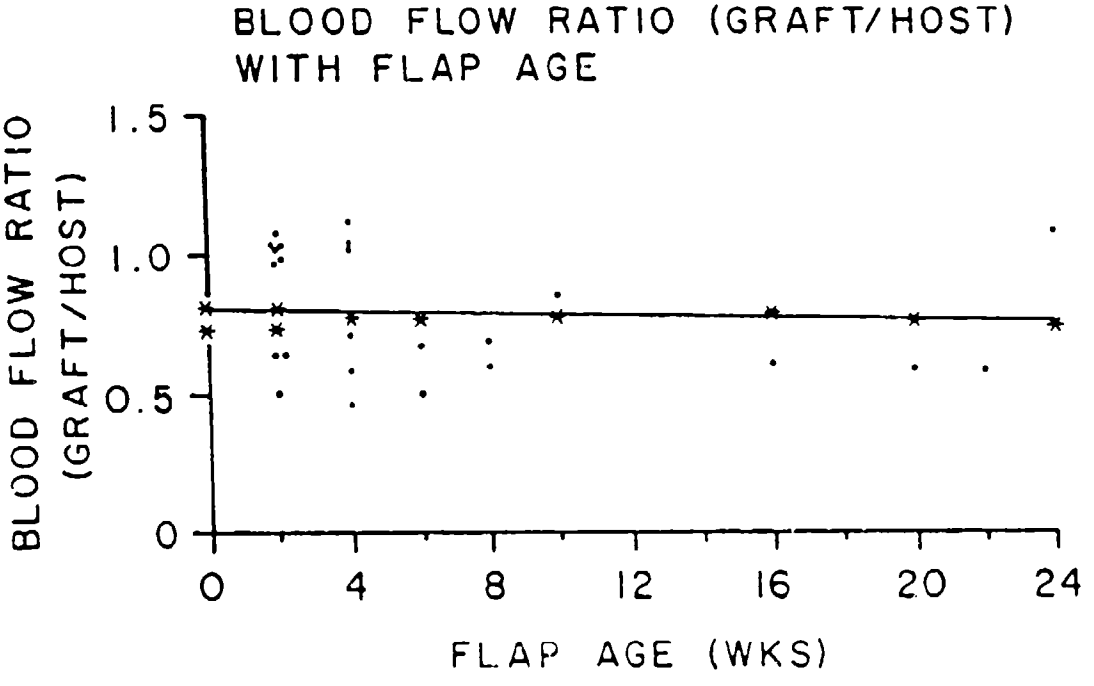

Figure 9. Blood Flow of the Graft Side vs. the Host Side of the Flap. Blood flow was compared as a ratio with age of the flap. Mean blood flow is not significantly altered as a function of age when a correlation coefficient is made, $r = -0.05$. Generally, the host side of the flap has greater blood flow, as measured with the LDV, than does the graft side.

examples, n=30, there was no change in the blood flow ratio, graft vs. host, with flap age. It was generally noted that the graft side of the flap had less blood flow (mean blood flow ratio graft/host = 0.80 at the end of stage III, 0 weeks, to 0.76 at the end of 24 weeks). There are eight cases (27%) in which blood flow on the graft side was greater than that on the host side. A correlation coefficient, however, demonstrated no significance (r = -0.05).

The variability seen in Figure 9 is not solely dependent upon either the graft or the host side. Similarly, these differences appeared unrelated to temperature, as body temperature was monitored throughout many experiments and showed no correlation with cutaneous blood flow. Temperature-dependent blood flow changes required greater shifts in temperature than were customarily encountered (0.5°C).

Effects of cyclosporine therapy on blood flow to the flap: Rat-rat flaps were investigated for changes in blood ratio in cyclosporine- and noncyclosporine-treated animals. The ratio of blood flow, graft vs. host, in the presence and absence of cyclosporine was unchanged (Table 4), the overall mean being 0.81±0.26. The blood flow ratio in human-rat flaps in the presence of cyclosporine was not significantly different from rat-rat flaps receiving cyclosporine.

Table 4. Blood Flow Ratios[1] in the Skin Sandwich Flap

Flap Type	+CS RX	-CS RX
Rat-Rat	0.85[2]±0.26 (7)[3]	0.80±0.25 (18)
Human-Rat	0.78±0.23 (12)	Not available

[1]Graft LDV value/host LDV value.
[2]Mean ± SD.
[3]Number of animals.
+CS RX = treatment with cyclosporine.
-CS RX = no treatment with cyclosporine.

Comparative analysis of blood flow to the rat-rat flap as a function of age, graft vs. host, and effect of cyclosporine: It is recognized that following skin injury, healing processes go on for a considerable period of time. Changes in blood flow may accompany that healing process. Therefore, alterations in blood flow to the graft and host sides of the flap, as measured by LDV, were investigated as a function of flap age.

In these experiments, as in most, the LDV was placed on the flap and left on throughout the entire experiment. An average LDV value was obtained from the experiment, converted to milliliters per minute (see Figure 8) with the LDV:EBFM correlation, and plotted as a function of flap age (see Figure 10). Flap age was determined from the end of stage III, e.g., at the first experiment, the flap age was 14 days.

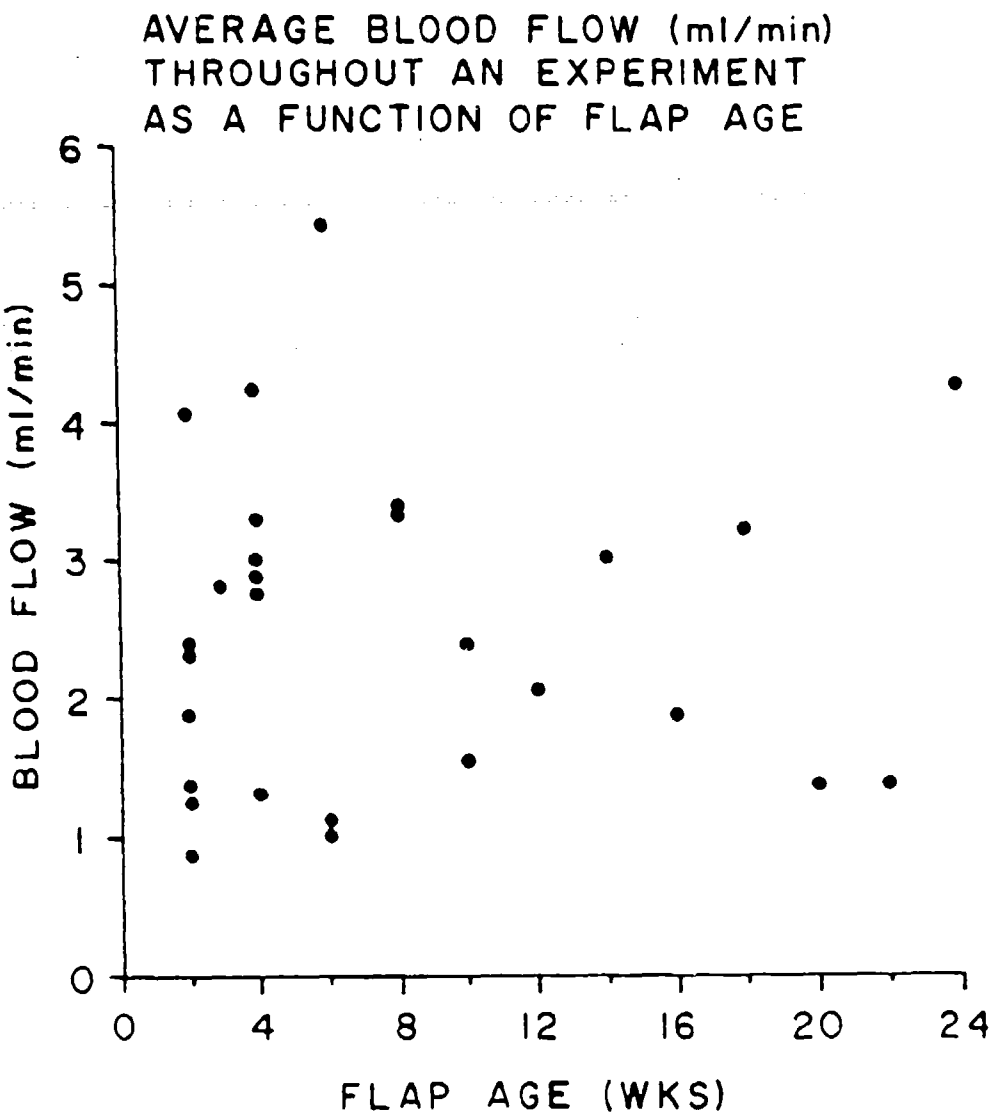

Figure 10. Average Blood Flow as a Function of Flap Age. Average blood flow is illustrated as milliliters per minute, calculated from the slope of values presented in Figure 8 during an experiment, as a function of flap age. Each point represents an individual flap at that particular flap age. After week 12, each point represents the same flap (Z1) at 2-week intervals. Note the high rate of blood flow in the old flap, age 24 weeks.

The number of flaps at any particular time period decreased as a function of flap age, seven at week 2, to one at week 12 and thereafter. Curiously, the animal at week 12 and thereafter had the same inherent variability as seen in individual animals before week 12. Furthermore, while the average blood flow was near 2 ml/min, there were experiments in which the average blood flow was in excess of 5 ml/min (see week 6 in Figure 10). The variability of blood flow within each age group may merely reflect the placement of the LDV probe. Probe placement over major vessels within the flap produced higher instrument readings than when placed 1-2 mm on either side of the vessel.

Percutaneous absorption

Application of [^{14}C]-benzoic acid (4.8 uCi) in ethanol to the host side of the rat-rat skin sandwich flap in a Holliseal well results in the rapid evaporation of vehicle with the deposition of 20 ug of the compound on the test site. The concentration of benzoic acid in the flap blood draining the test site steadily increases and reaches a plateau at approximately 1 hr (Figure 11). The concentration of benzoic acid in systemic blood taken from the contralateral femoral vein also steadily rises throughout the experiment, but to a much lesser extent than seen in flap blood. At 2 hr, the concentration of benzoic acid in flap blood is 30 times greater than in systemic blood.

The percutaneous absorption of benzoic acid in an aqueous vehicle has also been studied. The Holliseal well fixed to the host side of the flap was filled with 100 ul of the isotonic saline solution containing benzoic acid (0.52 uCi/ul). The well was covered with Parafilm to prevent evaporation. The concentration of benzoic acid in flap (Figure 12A) and systemic blood (Figure 12B) raised to a maximum at approximately 30 min following application and then gradually declined. The concentration of benzoic acid in flap blood was again 30 times greater than benzoic acid concentration in systemic blood. Two weeks later, the Holliseal well was placed on the grafted skin side of the sandwich flap and the experiment repeated on the same rat. Again, the concentration of benzoic acid in flap blood was 30 times greater than in systemic blood, and the area under the curve was the same as that noted in the experiments illustrated in Figure 12, A and B.

In both experiments, the concentration of benzoic acid in the silicone well dropped from 2.2 ug/ul at t=0 to 0.75 ug/ul at 3 hr. The figures mentioned above are representative of typical experiments.

Unless flux is extremely high and blood flow is extremely low, microcirculation in the skin is believed to have a negligible effect on the percutaneous absorption processes (1). Certain agents have high rates of flux, such that enough agent is able to cross a small area of skin and produce a systemic effect, e.g., nitroglycerin and certain chemical warfare agents. Assessment of percutaneous absorption following an alteration in the microcirculation was accomplished by applying local iontophoresis, 0.5 mA for 15 min, to a 1 mM solution of phenylephrine in distilled water. The treatment produced a local vasoconstriction in the flap, lasting 30 min. Control experiments utilized iontophoresis of distilled water alone or no iontophoresis.

A comparison of blood flow during each of the above experiments (as measured by the LDV) is illustrated in Figure 13. Following iontophoresis of phenylephrine, there was a rapid reduction (>50%) in microcirculation to the skin which began to recover within 20-30 min and normalized by 2-3 hr.

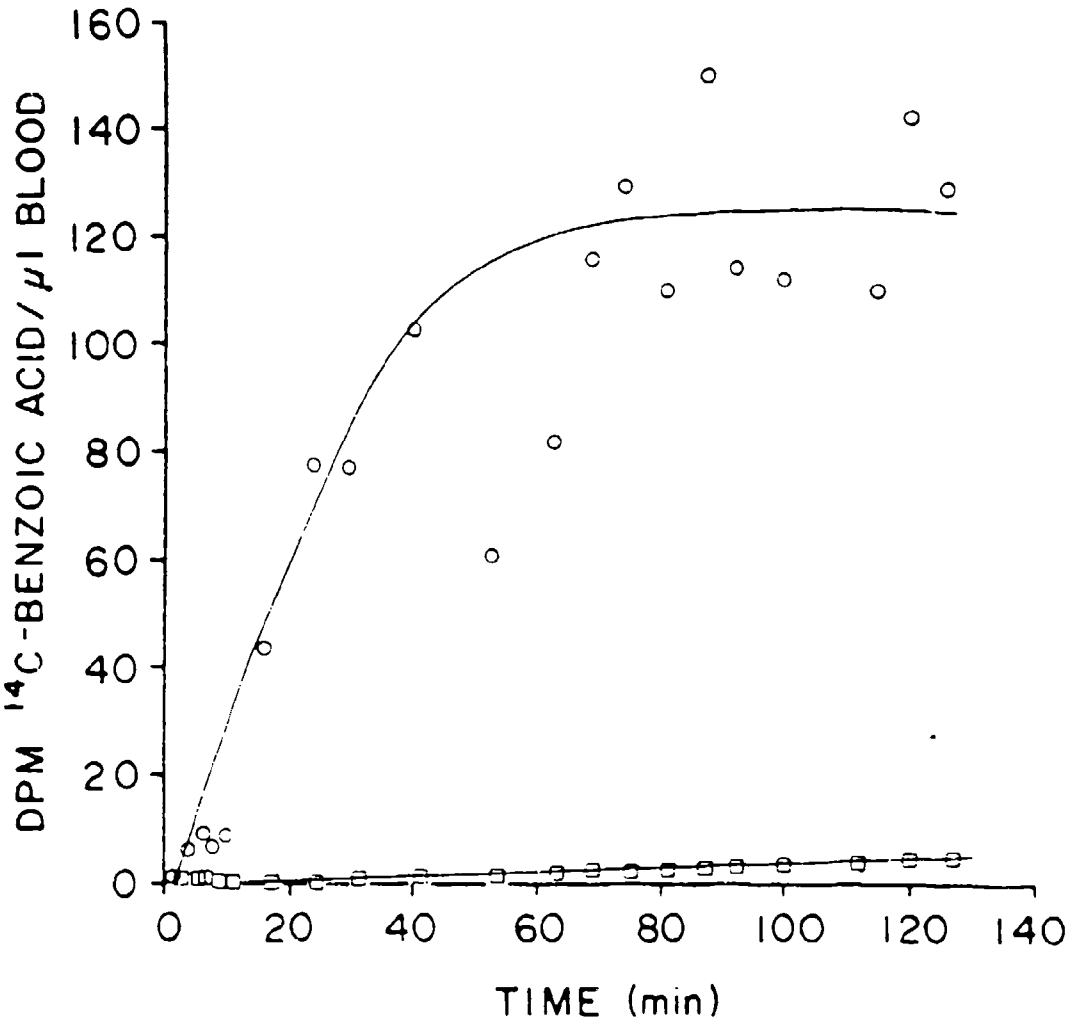

Figure 11: Transcutaneous Absorption of [^{14}C]-Benzoic Acid. Representative experiments of the transcutaneous absorption of [^{14}C]-benzoic acid applied to the flap in an ethanol base. 0 = dpm in flap blood; □ = dpm in systemic blood.

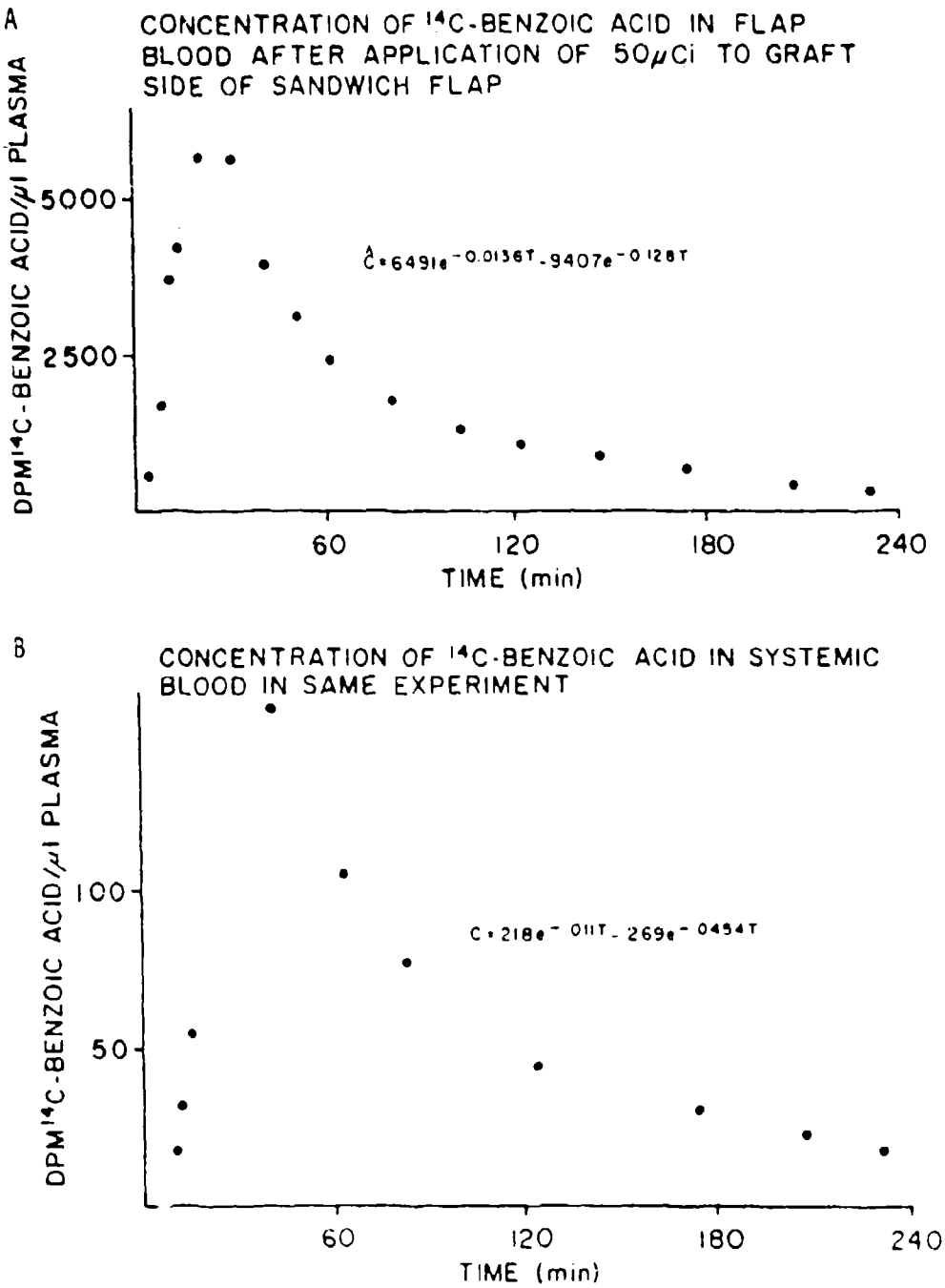

Figure 12: Concentration of [^{14}C]-Benzoic Acid in Flap Blood and Systemic Blood. Concentration of [^{14}C]-benzoic acid in flap blood (A) and in systemic blood (B) following the application of an aqueous preparation of benzoic acid to a chamber on the graft side of the skin sandwich flap. Values are cpm in blood as a function of time.

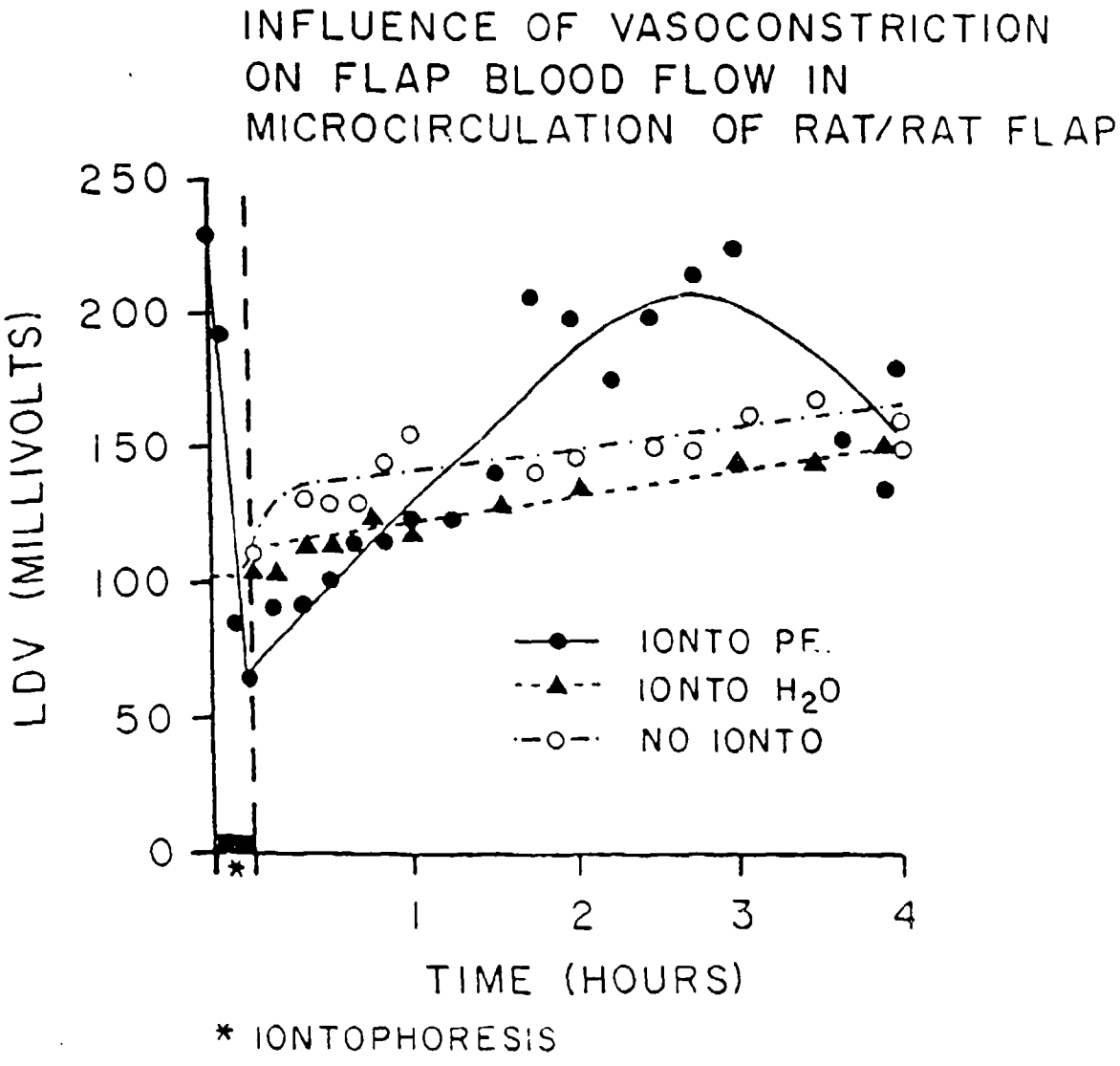

Figure 13. Influence of Vasoconstriction on Flap Blood Flow in Microcirculation of the Rat-Rat Flap. Plotted values represent LDV readings after iontophoresis of 1 mM phenylephrine, 15 min at 0.5 mA. These values are compared with that of iontophoresis of water alone, and both are compared to no iontophoresis. Each point represents a LDV reading at that time point.

Not displayed in this experiment is the frequent observation of rebound, i.e., blood flow after recovery being higher than that at baseline. The iontophoresis of water and the no iontophoresis components of these experiments demonstrated a slight increase in blood flow as a function of time. This increase was not unusual in any of the experiments, a appeared to not be significant. The same flap was used for each of these experiments, the experiments being conducted at 2-week intervals. The difference in baseline again represents the variability previously addressed.

Figure 14 demonstrates the flux of [^{14}C]-benzoic acid across the grafted surface of a rat-rat skin flap following iontophoresis of water or the iontophoresis of phenylephrine. These fluxes were compared with those of benzoic acid without iontophoresis. Peak flux of benzoic acid alone across grafted rat skin is approximately 1 ug/cm^2/min. Iontophoresis of distilled water or 1 mM phenylephrine in distilled water enhanced peak flux 5- and 12-fold, respectively, above that observed in the passive benzoic acid experiments. Iontophoresis of distilled water produced an increase in flux of benzoic acid across rat grafted skin at early time periods (<1 hr) above benzoic acid alone or following iontophoresis of phenylephrine. The reduction of detectable benzoic acid in flap blood following iontophoresis of phenylephrine, compared with the flap blood concentration following iontophoresis of water, reflected the influence of vasoconstriction produced by phenylephrine on the percutaneous absorption of benzoic acid.

While a steady state in flux was reached in benzoic acid alone and iontophoresis of water pretreatment experiments, the profile of benzoic acid absorption following iontophoresis of phenylephrine was dramatically different. Following the dissipation of vasoconstriction, a hyperemia was noted, resulting in a peak flux 12-fold greater than benzoic acid alone and 2.4-fold greater than pretreatment with iontophoresis of distilled water. Thereafter, the flux in the phenylephrine-pretreated flaps decreased to zero at the end of 4 hr.

The importance of aging on the percutaneous absorption of benzoic acid across grafted rat skin in the flap model system was investigated using the same flap over a period of 16 weeks (Figure 15). While a considerable difference in the absorption profile of benzoic acid was noted when blood flow was assumed to be 1.5 ml/min throughout each experiment, there was no correlation between flap age and percent of original dose absorbed. Previous experience suggests that flap blood flow is critical to the absorption of benzoic acid. Blood flow as monitored by LDV in the above experiments was highly variable. Correction of the percutaneous absorption profiles with flap blood flow at each time period of blood collection transforms the data into profiles that are very similar.

Comparison of caffeine and benzoic acid absorption across grafted and nongrafted skin: The percutaneous absorption of [^{14}C]-benzoic acid and [^{14}C]-caffeine across both sides of the flap model was compared to illustrate any differences between grafted and nongrafted rat skin (Table 5). The percent of absorption of the original dose of benzoic acid across rat graft and host was not significantly different, graft vs. host. Approximately 50% of the original caffeine dose was percutaneously absorbed across both rat graft and host (nongrafted) sides of the flap, while only 12% of the original dose was absorbed across a human graft. Similarly, no significant difference was found in the absorption of benzoic acid across grafted (10%) and nongrafted (6%) skin.

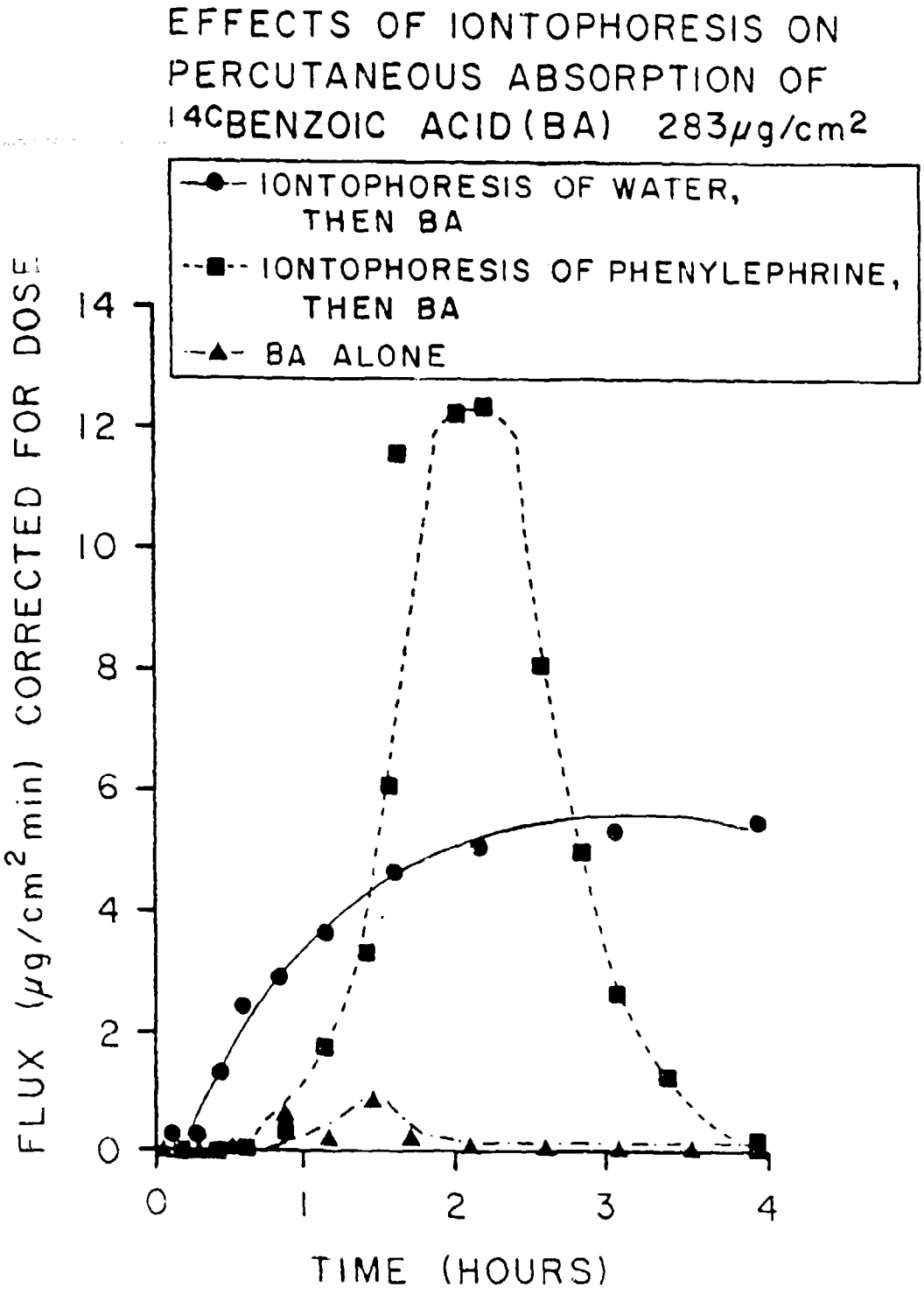

Figure 14. Effects of Iontophoresis on Percutaneous Absorption of [^{14}C]-Benzoic Acid. Values represent the mean of two experiments. Each experiment was performed on the same animal at 2-week intervals, i.e., benzoic acid in buffered vehicle alone, with repeat of the experiment 2 weeks later. Prior to the application of benzoic acid, the flap graft was treated with iontophoresis of water or phenylephrine for 15 min (see Materials and Methods). Data are presented as flux corrected for the dose applied.

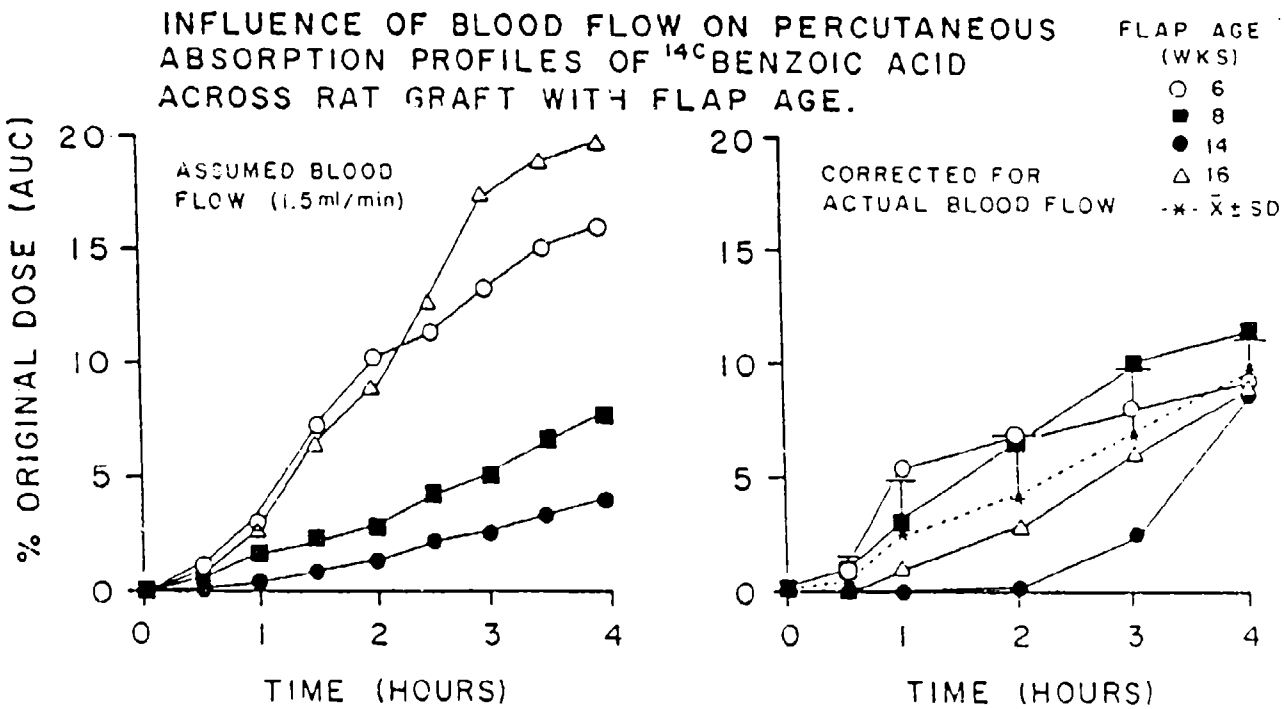

Figure 15. Influence of Blood Flow on Percutaneous Absorption Profiles of [^{14}C]-Benzoic Acid across the Rat Graft with Flap Age. Data are presented as a percent of the original dose absorbed. This experiment was performed on the same animal at an average of 2-week intervals (see above). To avoid confusion of lines, the lower half of the standard deviation bar is not included for mean percent absorption of original dose.

Table 5. Percutaneous Absorption Across Graft and Host Sides of the Flap

Percent Original Dose[1]	Rat Graft	Rat Host	Human Graft
[^{14}C]-caffeine[2]	49.4±3.8 (2)[3]	51.5±1.2 (3)	11.9±2.6 (6)
[^{14}C]-benzoic acid[4]	9.7±1.6 (6)	5.6±2.5 (3)	Not done

[1] x ± SEM.
[2] 35 ug/cm^2 finite dose in 1.0 cm^2, with ethanol vehicle allowed to evaporate.
[3] Value in parentheses = number of individual experiments performed.
[4] 220 ug/cm^2 infinite dose in PBS, pH 6.0, exposed to 1.0 cm^2 skin.

Percutaneous absorption in vivo vs. in vitro: Human skin from the same source, 0.5 mm in thickness, was applied to a Franz cell at 37° or utilized for the generation of a sandwich flap. A dose of 36 ug/cm^2 of [^{14}C]-caffeine in ethanol (specific activity = 47.5 mCi/mM) was deposited into a Teflon well in both model systems and the ethanol vehicle allowed to evaporate. The percutaneous absorption of caffeine across human skin in vivo and in vitro (n=3) is illustrated in Figure 16. Significantly higher amounts of caffeine (14X) were detected in vivo than in vitro at 1 hr. After 4 hr, 2-fold more caffeine was detected in vivo than in vitro. A second dose of caffeine was applied in vivo (just prior to 2 hr, at which time steady state was developing) (Figure 17). A second dose was applied in vitro at 3 hr. The deposition of a single dose of caffeine (36 ug/cm^2) in vivo and in vitro results in the absorption of approximately 10% and 5% of the original dose, respectively, by 4 hr. The total amount of caffeine absorbed in vivo after two 36-ug dosages of caffeine was approximately 96% of the second dose. However, the percent of original dose absorbed 4 hr after one or two doses of caffeine in vitro did not alter significantly.

Tissue binding of caffeine in vivo and in vitro: The foregoing suggested that caffeine may bind within the skin in both model systems. To address this possibility, biopsies (2 mm) were obtained from both systems 4 hr after the second drug dose. Total radioactivity in the flap blood or receiving chamber and tissue following one and two doses of caffeine from the in vivo and in vitro model systems is presented in Table 6.

Four hours following one dose of caffeine in vivo, 3 ug/cm^2 had passed into flap blood, while 0.3 ug/cm^2 was detected within the skin. Following two doses of caffeine in vivo, 10-fold more drug was detected in the flap blood, yet the amount in the skin did not differ from that observed after a single dose. The in vitro model system produced a markedly different profile. The amount of caffeine in the receiving chamber following one or two doses was the same, while the amount within the skin increased 11-fold following two doses.

Assessment of model system for studying metabolism of percutaneously applied agent

Assessment of the capacity of the skin sandwich flap system to metabolize percutaneously applied agents was investigated with topical vidarabine (ara-A), an antiviral agent. While this agent is not readily absorbed through an

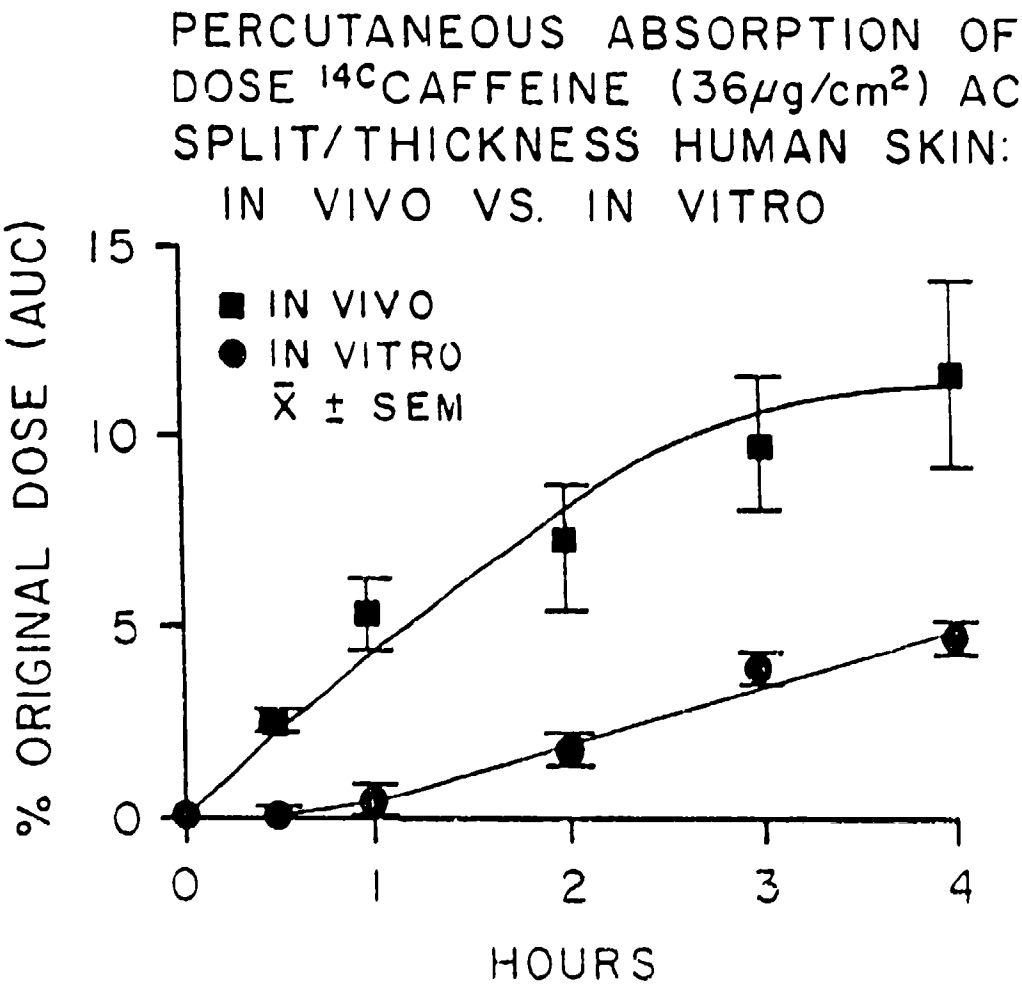

Figure 16. Percutaneous Absorption of a Single Dose of [^{14}C]-Caffeine. Comparison is made of percutaneous absorption of caffeine across a split-thickness human skin graft, in vivo vs. in vitro. Values represent the mean of triplicate experiments, each performed with a different skin source. However, the in vivo skin source and the in vitro skin source for any one experiment are from the same donor (the in vitro experiments were done prior to the in vivo experiments). Data are presented as percent of the original dose absorbed, area under the curve (AUC).

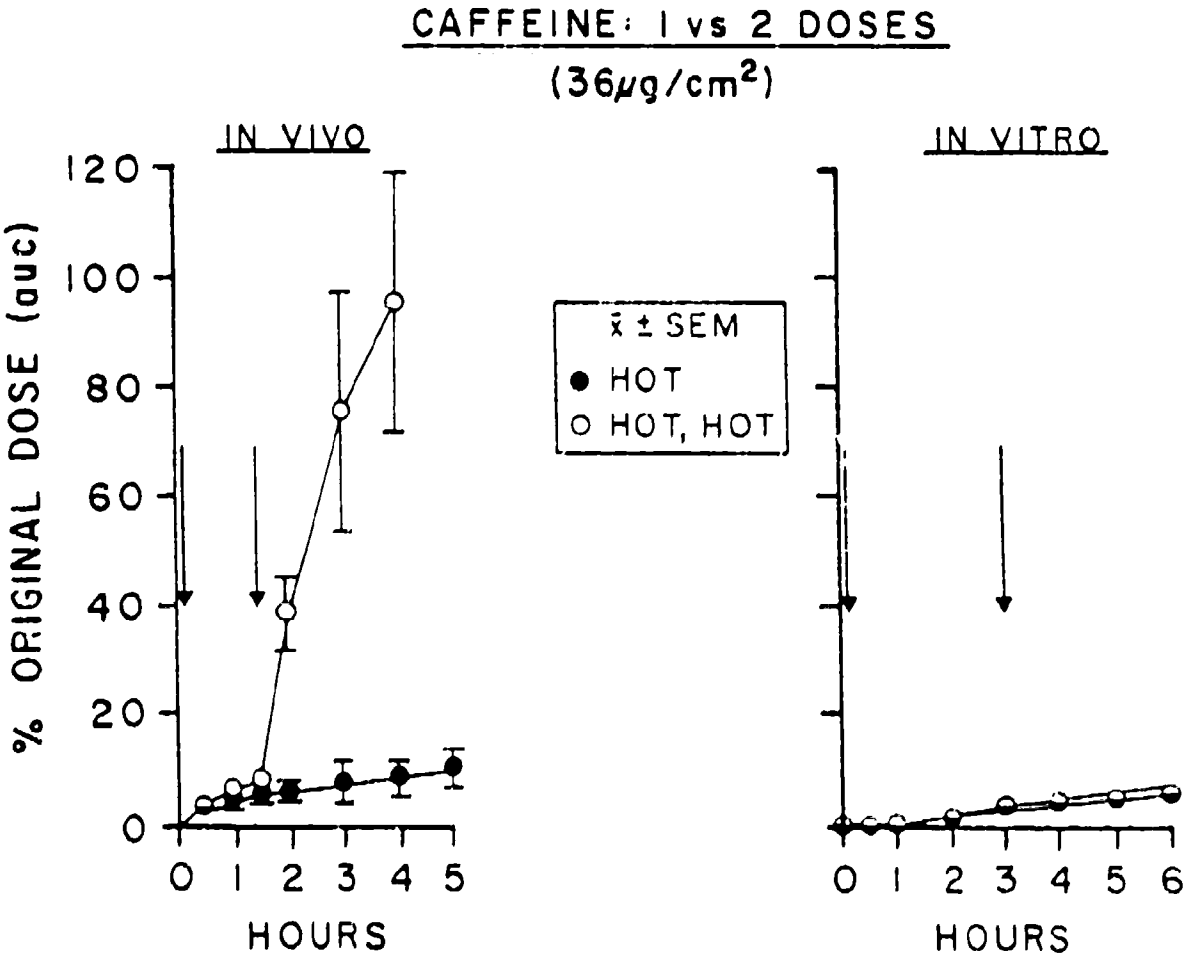

Figure 17. Comparison of Application of a Standard Dose of [^{14}C]-Caffeine. Comparison was made of application of caffeine in ethanol (see Materials and Methods) to human skin in vivo or in vitro. Less than expected amounts were noted in vitro; hence, a second dose was added at the time indicated by the arrow (both in vivo and in vitro). Both dosages were radioactive. In the in vivo setting, as a separate experiment, a second dose was added at 1.5 hr. This resulted in a prompt increase in the total amount of the original dose absorbed. A similar phenomenon was not observed in vitro.

Table 6: [^{14}C]-Caffeine in Skin and Blood following One and Two Doses In Vivo and In Vitro

	ug/cm^2			
	In Vivo[1]		In Vitro[2]	
	Flap blood	Skin	Chamber	Skin
1 dose[3]	2.8 (9.4)[4]	0.3 (0.9)	12.0±1.1[5] (21.4)	5.0±1.9 (4.4)
2 doses[6]	22.0 (30.5)	0.2 (0.2)	11.9±2.3 (10.6)	57.0±14.2[7] (59.7)

[1]Grafted split-thickness (0.5 mm) human abdominal skin-rat skin sandwich flap 4 hr after drug deposition.
[2]Human abdominal skin (0.5 mm) on stirred 37°C Franz cell 4 hr after drug deposition.
[3]36 ug/cm^2.
[4]Percent original dose.
[5]x ± SEM (n=5).
[6]72 ug/cm^2.
[7]x ± SEM (n=4).

intact stratum corneum, it is a good molecule to study because it is thought to be converted to a sole inactive metabolite, ara-H, by adenosine deaminase in skin. The in vitro experiments consisted of measuring the flux of [^{3}H]-ara-A and [^{3}H]-ara-H across tape-stripped nude rat skin mounted on a diffusion cell at 37°C. Backflux of [^{3}H]-ara-H into the donor chamber was also monitored. A half-cell diffusion chamber was affixed to a tape-stripped skin sandwich flap for the in vivo experiments. [^{3}H]-Ara-A was placed in the half-cell diffusion chamber and aliquots of flap and systemic blood were periodically obtained and analyzed for [^{3}H]-ara-A and [^{3}H]-ara-H. The concentration of [^{3}H]-ara-H resulting from backflux and the concentration of [^{3}H]-ara-A in the donor chamber were also monitored.

Four parameters were measured in the in vitro and in vivo experiments: 1) permeability coefficient, 2) lag time of absorption, 3) percent ara-A metabolized by the skin, and 4) ara-H backflux. These data are presented in Table 7. The percent of ara-A metabolized by the skin and the amount of ara-H backflux in vitro appeared to be slightly greater than in vivo. These differences, however, were not significant.

Assessment of feasibility of transplanting hair-bearing skin to the nude rat

Previous experiments have revealed that an increased amount of topically applied agent is absorbed when applied to skin relatively rich in hair follicles (rat vs. human skin). Evaluation of the influence of hair follicle density in skin on percutaneous absorption necessitates a model with grafted hair follicle-rich skin. The initial feasibility of producing hair growth in skin grafts was tested using split-thickness skin grafts of scalp skin (STSG-SS) (0.4 and 1.0 mm thick) that were orthotopically grafted to nude rats. The rats received cyclosporine (11 mg/kg/day) in their drinking water.

Table 7. Comparison of In Vivo and In Vitro Diffusion and Metabolism of Vidarabine

Parameter	In Vitro (n=4)	In Vivo (n=2)
Permeability coefficient (cm/sec)	$2.35 \pm 0.17 \times 10^{-6}$	$2.75 \pm 0.39 \times 10^{-6}$
Lag time (min)	22.1 ± 1.2	21.0 ± 1.4
% Ara-A dermal metabolism[1]	71.4 ± 1.3	62.6 ± 0.7
Normalized backflux ara-H (cm/sec)	$1.95 \pm 0.21 \times 10^{-5}$	$1.70 \pm 0.14 \times 10^{-5}$

[1]Values refer to the amount of absorbed [3H]-ara-A that is metabolized to ara-H in the skin.

Two stages of hair growth were noted in rats grafted with STSG-SS, n=5, immediate and delayed. Hair growth was quite dense and occurred in the apparent absence of papillae in the immediate stage (the first 6 weeks of engraftment) (see Figure 18). The hair in this growth phase could be removed with very gentle tugging and fell out spontaneously at 6-8 weeks. Hair growth in the delayed stage (occurring 2-3 months after engraftment) was less dense. This hair could be removed only with firm tugging. There was histologic evidence of formation of new papillae in the delayed stage, and the density of hair in this stage correlated with the thickness of the scalp skin grafts. New hair appeared on an average of 1/cm^2/week in the thick (1-mm) grafts. The hair density by 10 weeks was 7.9 hairs/cm^2. The density of hair in the thinner (0.4-mm) grafts at 10 weeks was 3.5 hairs per cm^2. This was significantly different ($p<0.05$) from that observed in 1-mm grafts. Ten weeks after grafting, the hair in the thicker grafts had a mean length of 4.4 mm, significantly greater ($p<0.001$) than that in the thinner grafts (1.7 mm). The average diameter of the hair shaft in both the thick and thin grafts was that of terminal hair, 0.05 mm, at the various times tested.

Animal and experimental statistics

Statistics on the loss of sandwich flaps and the number of experiments performed per flap have been collected over the 3rd year of the project (December 1984 through December 1985) to highlight the problem areas in the generation of the flap (Table 8). Data presented were analyzed according to: 1) the total number of flaps generated per quarter (rat-rat and human-rat), 2) the total number of flaps lost to flap chewing or graft failure, and the stages during flap generation (I, II, III, and >III) in which the chewing of graft failure occurred, 3) the number of experiments per flap (1, 2, 3, 4, and >4), and 4) the total number of experiments performed per quarter. The two latter categories differentiate between experiments on flaps generated within that quarter and those that survived to be utilized experimentally at times outside that specific quarter. The number of experiments per quarter may, therefore, be less than the total number of experiments performed per flap.

Figure 18. Photograph of Hair Growth in a Human Split-Thickness Skin Graft on a Nude Rat. This photograph illustrates hair growth 6 weeks following orthotopic engraftment of human scalp skin (1.0 mm thick) to the lateral thoracic cage of the nude rat. The average length of hair was 1.0 cm (see Materials and Methods).

Table 8: Animal Statistics

Date	Total Flaps	Flaps Chewed						Graft Failure						Exp./Flap					Exp./Qtr.	
		I	II	III	>III	T	%T	I	II	III	>III	T	%T	1	2	3	4	>4	T	T
Dec 84-Mar 85																				
Rat-Rat	4	0	0	1	1	2	50	0	0	0	1	1	25	0	0	0	0	0	0	0
Human-Rat	58	2	0	0	9	11	19	4	3	2	15	24	41	12	3	0	0	0	18	17
Total	62	2	0	1	10	13	21	4	3	2	16	25	40	12	3	0	0	0	18	17
Apr 85-Jun 85																				
Rat-Rat	16	2	0	1	5	8	50	1	3	0	0	4	25	2	2	0	1	1[1]	22	6
Human-Rat	26	3	1	2	5	11	42	0	2	2	5	9	35	1	2	0	1	0	9	10
Total	42	5	1	3	10	19	45	1	5	2	5	13	31	3	4	0	2	1[1]	31	16
Jul 85-Sep 85																				
Rat-Rat	11	2	1	1	1	5	45	2	0	1	0	3	27	0	2	0	0	0	4	15
Human-Rat	24	1	5	3	0	9	38	2	0	4	1	7	29	3	1	1	0	1[2]	13	4
Total	35	3	6	4	1	14	40	4	0	5	1	10	29	3	3	1	0	1[2]	17	19
Oct 85- Dec 85																				
Rat-Rat	11	2	2	2	1	7	64	0	0	0	0	0	0	2	1	0	0	1	9	15
Human-Rat	30	3	2	2	1	8	27	3	10	0	4	17	57	2	1	0	0	0	4	12
Total	41	5	4	4	2	15	37	3	10	0	4	17	41	4	2	0	0	1	13	27

[1] Twelve Experiments

[2] Five Experiments

The total number of flaps generated each quarter remained about the same over the four quarters, approximately 40 for each quarter: approximately 12 rat-rat flaps and 27 human-rat flaps. The number of human-rat flaps generated depended upon experimental needs and the availability of human skin from elective plastic surgery procedures. Skin availability remained relatively stable over the time analyzed.

The percent of rat-rat flaps generated that were lost to flap chewing remained relatively stable at about 50%. Flap chewing of the human-rat flaps decreased from 42% in the second quarter to 27% in the fourth quarter. Flap chewing continued to be a problem which we specifically addressed over the last few months of the final year of the project; this may have accounted for the decrease to 27%. Regardless of the apparatus designed, the animals were still able to chew at the flap. The chewing behavior was not predictable and was not necessarily related to graft rejection. Some animals go for prolonged periods of time without chewing at their flaps. For example, one animal, 21 with a rat-rat flap, was used experimentally on 12 separate occasions without ever chewing at the flap. The use of metal collars around the rat's neck prohibits chewing but also inhibits grooming behavior which often leads to illness and death. The course of prevention currently in use involves more frequent changing of the restraining bandage and allowing time between bandaging for animal grooming. Other restraining systems are continually evaluated. These do not include tranquilizing drugs. The use of these drugs may interfere with metabolism studies to be done in the future. Although a search for new methods to inhibit the chewing behavior is continuous, it may be a reality that a certain percent of flap loss due to chewing will be inevitable. Our goal has been to reduce that percentage as much as possible.

The percent of total rat-rat flaps generated which were lost to graft failure decreased from about 25% in quarters I, II, and III to 0% in quarter IV. Although this decrease was dramatic and ideal, the percent of graft failure most probably will not be maintained at that level. The loss of human-rat flaps due to graft rejection decreased slowly from 41% in the first quarter to 29% in the third quarter. Closer examination of the failure data revealed that the majority of failures in the first quarter occurred after stage III (15 animals). At that time, animals were receiving 20 mg/kg of cyclosporine subcutaneously on day 1, and thereafter received 12.5 mg/kg of cyclosporine subcutaneously every other day through day 21. Maintenance dosing consisted of 12.5 mg/kg subcutaneously once per week for the duration of flap survival. These studies reflected an inadequate maintenance dose. Therefore, the dosing regimen was altered to 20 mg/kg of cyclosporine subcutaneously on day 1, followed by 12.5 mg/kg subcutaneously every other day through day 14 and maintaining cyclosporine in the drinking water at a dose of 11 mg/kg/day. The new dosing regimen with an enhanced maintenance dose reduced the incidence of graft rejection in the later stages of the flap generation (>stage III), but increased the number of grafts rejected in stages I and II. The shift in graft rejection to earlier stages suggests that the rejection in these stages was due to poor initial vascularization of the graft.

Flap losses in quarter IV due to graft rejection increased to 48% of the total human-rat flaps generated. The increase reflected the type of skin used for grafting. During the fourth quarter, human face skin was grafted as well as the usual human abdominal skin. Graft failure of the human face skin was initially much greater than that of abdominal skin grafts. Technical

difficulties associated with grafting multiple pieces of face skin to the rat sandwich host skin were circumvented by suturing small pieces of face skin together prior to grafting.

The most significant improvement in the flap statistics lay in the number of experiments performed per flap. In the first quarter, the majority of flaps were utilized in only one experiment (n=11). In the second, third, and fourth quarters, however, many animals with both rat-rat and human-rat flaps were utilized in two or more experiments. One particular rat-rat flap, which was generated in the second quarter, was used on 12 separate occasions over a period of 5 months. The average number of experiments performed per flap is approximately three for both rat-rat and human-rat flaps.

The final category, the total number of experiments per quarter, reflected the productivity of a two-person team involved in the generation, experimentation, and maintenance of the flaps. In general, 17 experiments were performed over the fourth quarter of the 3rd year, an average of 1.4 experiments per week. Of course, this was directly dependent upon the number of flaps available for experimentation, which varied from week to week. In the last quarter of that year (Oct 85-Dec 85), 27 experiments were performed. This was the result of experimentation on animal flaps generated in that specific quarter, as well as in previous quarters.

Conclusions/Discussion

The feasibility of successfully grafting split-thickness (0.5 mm) human skin onto a congenitally athymic (nude) rat in a skin sandwich flap with an isolated but accessible vasculature was accomplished. This achievement, however, was not as straightforward as initially anticipated. Although STSG from allogeneic rats revealed no evidence of graft rejection in this laboratory or that of others (4) over a 4-month period, 89% (50/56) of nude rats grafted with HSTSG slowly rejected their grafts between the 3rd and 6th week of engraftment.

Presently, cyclosporine therapy is utilized to prevent rejection of the human skin graft. Administration of cyclosporine involves an initial subcutaneous dose of 20 mg/kg on day 1 of surgery, when the HSTSG is grafted, followed by 12.5 mg/kg every other day through day 21. Cyclosporine therapy, therefore, was administered subcutaneously throughout stage I and II. Following the microvascular surgery and translocation of the flap to the dorsum of the rat (stage III), the animal received cyclosporine (11 mg/kg/day) in drinking water (sterile, pH 2.5). This dosing regimen significantly reduced the number of flaps lost to graft failure. Among flaps generated, approximately 26% of the flaps were lost due to chewing by the rat, and 35% were lost as a result of graft failure. Thus, approximately 39% of human-rat flaps that were initiated reached experimental stage. The likelihood, however, of a flap being reutilized for three or more separate experiments increased substantially, such that the average number of experiments has increased from 17 per quarter in the early quarters to 27 in the final quarter.

Incidence of graft rejection

The rejection of HSTSG by nude rats is an enigma. Festing et al. (34) suggests that this rejection depends on factors such as the size of grafts and the genetic background of the nude rats. Our data give no indication that such

factors play a role in the rejection. Three different strains of nude rats have been utilized and none showed evidence of selective rejection or acceptance of the HSTSG (data not presented). The diameter of the HSTSG also does not predict success or failure; however, thickness of the graft is a factor. HSTSG that are more than 0.7 mm thick will generally not survive the engraftment process and show early signs of failure. This contrasts with the early acceptance and later rejection we report herein.

Despite studies that indicate an absence of cell-mediated immune responses in nude rats (10,34,35), the present observations suggest that an immunologic mechanism is important to the rejection process. The histologic features of the host vs. graft reaction that occurs and the observation that regrafted rats rejected skin more rapidly than those who were grafted for the first time support this possibility. The blunted but positive mixed lymphocyte reaction with lymphocytes from the draining lymph nodes, but not from spleens, of nude rats that have rejected a HSTSG is interesting in two regards. First, it provides additional support that this curious rejection phenomenon is mediated immunologically; second, it supports the growing notion that skin has inherent immunologic properties (30). The cytotoxic response of lymphocytes of draining lymph nodes of the rats which have rejected HSTSG to human peripheral mononuclear cells is likewise curious and in harmony with the rest of the present observations. A population of lymphocytes bearing T-cell markers has been demonstrated in congenitally athymic rats and mice (11,36). The possibility that grafting with human skin reconstitutes thymic competence seems unlikely because nude rats do not respond well to concanavalin A (con A), phytohemagglutinin (PHA), or to tuberculin.

The present study indicates that humoral immunity plays an important role in HSTSG rejection. Commensurate with this observation are the studies which indicate that nude rats do not necessarily have an abnormal humoral response system (10,11,35,37). Rats that have previously rejected HSTSG have circulating IgG and IgM antibodies, which bind to target structures in the dermis (blood vessels) and epidermis (BMZ) of never-grafted normal human skin at a frequency less than that seen when grafted human skin from nude rats was used. The low incidence of successful engraftment of HSTSG precludes a detailed analysis of this phenomenon. The direct analysis of these successful grafts for deposition of immunoreactants prior to removal showed no binding of IgG or IgM to the BMZ or to the blood vessels. Nevertheless, the data do demonstrate that this skin, as a substrate, is more likely to bind the circulating immunoglobulins to the aforementioned structures which develop as rats reject the HSTSG. Human skin from grafts on nude mice did not show enhanced binding and thus was like never-grafted skin (data not presented). This raises the possibility that the antigenic component of the BMZ which binds circulating IgG from rats undergoing rejection is somehow more readily expressed during engraftment on the nude rat.

The antibodies which develop to the BMZ and to the vessels are likely to be the component which results in the delayed rejection described herein. This is based upon the following observations:

1. Appearance of immunoreactants in the BMZ and the superficial vasculature of the HSTSG undergoing rejection
2. Injection of serum from rats, who previously rejected grafts, into successfully grafted rats results in the prompt (7-9 days) rejection

of the previously successful HSTSG
3. Presence of IgG in the serum of rats undergoing rejection of HSTSG which binds to the same anatomic structures in human skin, BMZ, and vessels, as seen in the direct immunofluorescence studies
4. Disappearance of these antibodies 5-7 weeks after the rejection process is complete, with the failure of these antibodies to appear in the cyclosporine-treated animals.

Lear et al. (38) described an increase in uncontrolled B-cell activity directed at skin allografts in normal rats who were lethally irradiated, thymectomized, and reconstituted with bone marrow. This observation negates the importance of mature T-cells in this process but ignores the possibility that skin grafts contain T-cell maturation abilities. Interleukin-1 (IL-1), which is involved in T-cell activity, has recently been reported (39) to be present in skin and thus may be a causative agent in the graft rejection process. This laboratory, however, has noted that skin allografts from unrelated rats (n=4) and xenografts from nude mice (n=3) to the nude rats were not rejected during the 4-month observation period (unpublished observations). It is possible that human skin might release specific T-cell maturation products such as thymus-like hormone (30). However, the fact that HSTSG on nude mice remain viable for the life of the mouse suggests that this is less likely. On the contrary, the selective enhancement of some cell-mediated components of the immune system in the draining lymph nodes of rats rejecting HSTSG does support such a notion. Reports of lymph node analyses from nude rats grafted with HSTSG have not been found. While graft size does not influence rejection (see above), it is possible that size does play a role in the partial restoration of cell-mediated responses of the draining lymph nodes. Analyses of the lymph nodes from nude rats accepting HSTSG, rat skin grafts, or nude mouse skin grafts have not been performed.

Of intrigue are the preliminary data of HSTSG that are first grafted to nude mice for 60 days and then transplanted to the nude rat (n=3). In the three experiments in which this has been accomplished, there has been no sign of rejection during the 3-month observation period, despite the fact that one of the grafts was placed on a rat that had previously rejected a HSTSG. The rejection phenomenon is further complicated by the observation that 5-10% of HSTSG on nude rats survive without treatments to prolong engraftment. It is unknown whether this is secondary to the host or to the lack of appropriate antigens in the graft. It is likely secondary to the host, as HSTSG from the same source can undergo either rejection or survival independent of other variables. Regrafting of those rats that had HSTSG survive for more than 90 days with a new HSTSG does not result in rejection of the second graft. The rarity of graft survival without treatment has precluded an experiment to determine whether the tolerance to HSTSG can be passively transferred with spleen cells. It is apparent that the process of rejection of HSTSG by nude rats is complicated, i.e. both the host and the graft appear to play critical roles.

Protocols were designed to prevent graft rejection by treating the graft or the animal before transplantation or the animals after grafting. Treatments directed at the graft before engraftment were designed to eliminate class II molecules, Ia-like, and migratory immunocompetent cells, passenger leukocytes. Class II molecules of human skin are present in Langerhans cell of the epidermis and are present on adnexal structures, endothelium, and dendritic

cells in the dermis (35,36). Anti-HLA-DR has been shown to bind to antigenic molecules of this type in mice and to render them inoperative in assays in which Ia is needed for stimulation (33). While anti-HLA-DR is effective in inhibiting immune responses where Ia-like activity is necessary (33,40), there is no evidence that skin treated with this antibody prior to transplantation remains bound and functional after grafting. Other unpublished experiments from this laboratory have shown that the anti-HLA-DR can be readily observed in the HSTSG by direct immunofluorescence 7 days after transplantation to nude mice if the transplanted HSTSG has been preincubated in anti-HLA-DR. Unfortunately, these experiments were designed for other purposes and were terminated at 7 days. Despite these observations, failure of this therapy to prolong graft survival supports the notion that class II antigens are probably not the definitive immunogens in settings in which the class I differences exist. This conclusion is in agreement with that of Steinmuller (41) in his comparative survival studies of skin grafts across similar types of antigenic differences. Failure of x-irradiation of the HSTSG to prolong graft survival on nude rats is similar to that seen by Steinmuller (41) but contrasts with the prolonged survival of other organs similarly treated before grafting (42).

Total body x-irradiation will prolong allografts in normal animals (43). The success of this treatment in prolonging graft survival after 21 days was predicted to be insignificant. However, it is unknown whether the amount of antigen(s) within the HSTSG diminished over the 2-week engraftment period and correlated with graft survival. While graft survival was lengthened over control, the differences were not significant.

Increased natural killer activity has been described in nude mice and nude rats (35). The recent report of anti-asialo GM blocking natural killer activity (27), and the possibility that this phenomenon has an effector role in the rejection process, prompted an experiment wherein rats were treated with anti-asialo GM sera prior to engraftment and monitored for the duration of graft survival. This treatment resulted in the death of three of the five grafted and treated rats within the first 3 weeks. In the two rats that survived, graft survival was not prolonged. Treatment of the recipient rats with ALS was toxic, resulting in two deaths before day 21, but did permit survival of the grafts until about 7 days after the injections were stopped in the two surviving animals. This suggests that the ALS therapy was merely inhibitory to the effector process and did not alter the recognition process. Treatment with BPT alone resulted in a lymphocytosis, but did not significantly enhance graft survival in the two rats receiving this therapy. While BPT injections are reported to enhance the effectiveness of ALS and cyclosporine in prolonging the survival of allografts (44), the preliminary data from two rats receiving both BPT (2 weeks) and cyclosporine (3 weeks) revealed no apparent enhancement of graft survival compared with those rats receiving cyclosporine alone.

Of the treatments tested, only cyclosporine prolonged the engraftment of HSTSG on nude rats (45). This suggests that immune mechanisms are active in the rejection process. Prolonged survival of HSTSG beyond 90 days on nude rats, as demonstrated in this study, resulted from short courses of injections of cyclosporine, 25 mg/kg, for 21 consecutive days, without maintenance injection doses thereafter. This short treatment period is in contrast to the long maintenance dose protocols required for survival of HSTSG on normal rats (46). Pinto et al. (28) demonstrated prolongation of skin graft

survival across different genetic barriers in normal rats with a relatively short course of cyclosporine (20 mg/kg for 14 days), which increased the mean graft survival from 14-16 days to 67 days. These same investigators demonstrated that a 6-day course of cyclosporine enhanced graft survival by only 3-4 days. In further experiments, they demonstrated that Bordetella pertussis vaccine before engraftment extended both the 6- and the 14-day effects of cyclosporine, whereas the vaccine alone had essentially no effect. In the present experiments, the effect of cyclosporine for 7 and 14 days extended the life of the grafts, but not in a significant way.

Homan et al. (47) showed that cyclosporine did not prevent the allograft rejection process in rats that had been sensitized with skin grafts. Discontinuation of cyclosporine treatments in rats regrafted following a primary sensitization results in prompt rejection of that skin allograft (44). The present data are in accord with these observations, with graft rejection occurring within 9 days after withdrawal of therapy. The mechanism by which a short course of cyclosporine prolonged allograft and xenograft remains unclear. However, cyclosporine injections for 7 and 14 consecutive days had no influence on survival of xenogeneic skin grafts. Furthermore, the rejection of HSTSG on flaps after day 21 caused us to implement low doses of cyclosporine for the entire period that the grafts were available for experimental use.

Assessment of blood flow to the flap

Blood flow is acknowledged to be a critical factor in percutaneous absorption (1), but to date has been unquantified. Studies of blood flow through the skin have been neglected due to the lack of a model system. Assessment of the blood flow to the flap has been investigated with a variety of instruments to analyze the extent of collateral circulation, changes in the flap blood flow after surgery, and differences in blood flow to grafted and nongrafted sides of the flap as a function of flap age.

Tissue anoxia was minimized throughout the generation of the flap to minimize collateral circulation (22). The presence of significant collateral circulation to the flap would have diminished the effectiveness of the model in quantifying the percutaneous absorption process. Success in minimizing collateral circulation to the flap was evident from the India ink study, which resulted in the appearance of ink throughout the flap with very little in the surrounding skin. Histologically, the ink was present in all arterioles, venuoles, and capillaries. This included the capillaries at the dermal-epidermal junction. Further evidence in minimal collateral circulation to the flap was assessed with a dermofluorometer, in which the collateral circulation was estimated to be less than 10% of the total blood supply to the flap via the superficial epigastric vascular system.

Flap blood flow after surgery

Data gathered from three different locations on a particular flap throughout stages II and III, in which the flap is raised from the belly and finally translocated to the rat dorsum, respectively, reveal that blood flow in the flap, as assessed by LDV, was not significantly different in the three areas following surgery. These data confirm that vascularization throughout the flap is complete and equitable.

Blood flow volume (millimeters per minute) to the flap has been quantitated with the EBFM. As stated above, this instrument utilizes a "C"

probe, which directly fits onto the artery supplying the flap. An analysis of eight different mature flaps revealed that the actual blood flow volume to the flap ranged from 1.5 to 2.0 ml/min.

Blood flow to the grafted and nongrafted sides of the flap with flap age

Further assessment of blood flow to the flap with the LDV was made, comparing blood flow to the grafted and nongrafted sides of the flap with flap age. The ratio of blood flow to the grafted:nongrafted sides of the flap revealed no significant change ($p>0.05$) with flap age. The variability in the ratios at any flap age was not solely dependent on a particular side of the flap. Similarly, these differences appear unrelated to temperature, as body temperature was monitored throughout the experiment. Temperature-dependent blood flow changes required greater shifts in temperature (2°C) than were customarily encountered during an experiment (±0.05°C).

Although the ratio of blood flow (graft/host) in the flap was not altered, the actual volume of blood flow to the flap appeared to change with age. Throughout an experiment, data demonstrate no correlation between the average blood flow volume to the flap and flap age. Considerable variation was noted in the blood flow in any particular flap on a week to week basis. This probably reflects the variability in LDV probe placement, which was not always in the same location on each flap. The blood flow in a particular area on the flap can vary as much as 50%, but the LDV probe was always positioned directly opposite the well into which the drug was dispensed.

Cyclosporine is routinely utilized in the generation of human-rat flaps to prevent graft failure. The influence of cyclosporine on blood flow in rat-rat flaps was not significant, since the ratio of blood flow in the presence and absence of cyclosporine was unchanged in the rat-rat flap (data not presented). The average blood flow ratio in the human-rat flaps did not differ significantly from that observed in the rat-rat flaps.

Validation of the flap for studies in percutaneous absorption

The flap model has been validated for studies in percutaneous absorption with: a) assessment of the drug concentration in both flap and systemic blood following absorption from either side of the flap; b) changes in absorption profiles using different vehicles; c) influence of flap blood flow and flap age on the extent of absorption; d) differences in percutaneous absorption of selected compounds in vitro vs. in vivo; and e) the use of this model to study oxidative metabolism by the skin (see Results/Discussion).

Correlation of flap blood flow by laser Doppler flow velocimeter, dermofluorometer, and electromagnetic blood flow meter

The LDV was used to monitor flap blood flow throughout each experiment. This instrument has a fiberoptic probe which noninvasively sits directly on the flap surface. The instrument was preferred in this laboratory because it does not interfere with the collection of blood from the flap. Whether the LDV does in fact provide a direct assessment of blood flow volume to the skin was investigated by correlating the LDV measurements with those obtained simultaneously from two other instruments, the DF and the EBFM.

The appearance and disappearance of fluorescein from the skin following an intravenous injection directly correlates with the capillary blood flow and renal clearance. The simultaneous noninvasive monitoring of dye appearance

into the flap by the DF and the velocity of red blood cells in the flap by the LDV produced a correlation coefficient of blood flow between the two instruments of r=0.95.

The analogue readout from the LDV is in millivolts. This is an awkward unit for describing the volume of blood per time in the flux equations. Therefore, it was desirable to correlate the LDV millivolt units with an instrument which measures actual blood flow volume (milliliters per minute). A new instrument, the EBFM, utilizes a "C-type" probe, which is directly attached to the artery supplying the flap. This instrument, although invasive, directly evaluates blood flow in volume per time and has been used to correlate LDV values of blood flow obtained simultaneously with actual EBFM blood flow rates. This type of direct correlation had never been made. The correlation coefficient between the two instruments is r=0.993. This strong correlation confirms that the LDV does in fact reflect actual blood flow to the flap. Even more important, this correlation lends itself to direct conversion of the LDV readings collected throughout an experiment to actual milliliters per minute. This unit simplifies the flux equation (concentration flap blood X flap blood flow/surface area X time) such that the actual amount (micrograms) of compound absorbed across the skin can be reported (ug/cm^2 * min).

In general, the transcutaneous absorption of a compound resulted in flap blood concentrations of drug that were 30-fold greater than the systemic blood concentration. The percutaneous absorption of benzoic acid or caffeine across the grafted or nongrafted sides of the flap did not differ significantly. Therefore, although the grafted side of the flap was initially 0.5 mm, which is approximately one-half the thickness of the host skin, the absorption profiles across both thicknesses were similar. The lack of disparity between split-thickness skin grafts and full-thickness host skin most probably reflects the influence of capillaries present at the dermal-epidermal junction in the skin of both sides of the flap. The presence of capillaries at this same depth within the skin would lessen the influence of dermis thickness, since the majority of compound diffusing across the stratum corneum and epidermis would be absorbed directly into the capillaries and then into the flap venous system.

Unless flux is extremely high and blood flow extremely low, microcirculation in the skin is believed to have a negligible effect on the percutaneous absorption process (1,46). Certain agents have high rates of flux, such that sufficient agent is able to diffuse across a small area of skin to produce a pharmacologic effect, e.g., nitroglycerine and certain chemical warfare agents. Assessment of percutaneous absorption following an alteration in the microcirculation was investigated by iontophoresing (0.5 mA, 15 min) the alpha agonist, phenylephrine, in distilled water (mM) across either the graft or host sides of a rat-rat flap. This treatment produced a local vasoconstriction producing a 50% or greater reduction in flap blood flow (as monitored by LDV), which persisted for approximately 30 min, and was typically followed by a hyperemic effect. Iontophoresis of distilled water produced no alteration in flap blood flow as measured by LDV from that observed in experiments with no iontophoresis, yet the flux of [^{14}C]-benzoic acid across the iontophoretically pretreated graft was 5-fold above that in the grafts receiving benzoic acid alone. Peak flux of benzoic acid across the phenylephrine treated graft was 12-fold greater than benzoic acid alone and 2.5-fold greater than the graft pretreated with iontophoresis with water. In these analyses, it is apparent that iontophoresis of water or phenylephrine changed the barrier function such

that greater amounts of benzoic acid were absorbed. Comparing flux as a function of time in these experiments revealed that phenylephrine altered absorption in a significant way. During vasoconstriction, it appeared that benzoic acid moved through the stratum corneum and epidermis and remained in the epidermis and/or dermis until the local microcirculation was restored. Thereafter, a rapid flux of benzoic acid into the bloodstream was noted. In these experiments, there was reflux hyperemia at 2 and 3 hr. Hyperemia may be the cause for the flux decreasing to zero at the conclusion of the experiment. The infinite dose of benzoic acid on the surface was sufficient to maintain a steady state appearance of benzoic acid such as seen with iontophoresis of water alone. If these experiments had been conducted for longer periods of time, it is likely that detectable amounts of benzoic acid would have been seen once again in the flap blood of the flap treated with phenylephrine. Although the effects of skin aging on percutaneous absorption has been studied by Roskos et al (48) in various human age groups, little is known about the influence of aging of grafted human skin on percutaneous absorption. Utilizing the same grafted rat skin flap over a period of 16 weeks revealed no correlation between flap age and the extent of benzoic acid passively absorbed at the end of 4 hr. There was, however, considerable variation in the extent of benzoic acid absorption between the experiments. Previous experience suggested that blood flow was critical in the percutaneous absorption of benzoic acid. Flap blood flow in the various experiments was assumed to be 1.5 ml/min. In fact, the flap blood flow in the experiments differed as much as 2-fold. Calculating the extent of benzoic acid absorption with flap blood flow at each time period of blood collection results in profiles of absorption that are very similar. Thus, including actual cutaneous blood flow as a factor in calculating equations reflecting percutaneous absorption dramatically altered the interpretation of experimental results. This analysis again confirms the necessity of understanding blood flow to the skin when attempting to accurately determine the amount of a compound absorbed following topical application.

Graft vs. host and in vivo vs. in vitro

A comparison of the percutaneous absorption across both sides of the flap model system revealed no difference in the percent of original dose of caffeine or benzoic acid across grafted or nongrafted rat skin. Theoretically, if percutaneous absorption across grafted and nongrafted rat skin does not differ, then percutaneous absorption across grafted human skin on the flap and human skin in vivo would also be very similar. Absorption of caffeine across grafted rat skin was approximately 50% of the original dose, while absorption across grafted human skin was only 12%. These data suggested that although the nude rat was, for the most part, hairless, the absorption profile of caffeine across this type of skin was quite different from that across human skin. The data serve to validate the system for percutaneous absorption studies and lend themselves to predict that the absorption of compounds across human skin in situ will be very similar to that seen across the human component of our skin flap system.

The ability to predict the in vivo percutaneous absorption of a compound from in vitro model systems is an ultimate goal, but to date is controversial. The percutaneous absorption of [^{14}C]-caffeine following deposition onto human skin was therefore compared in vivo using the human-rat flap model and in vitro using human skin on a Franz cell. Significantly greater quantities of caffeine were detected at 1 and 4 hr (14-fold and 2-fold, respectively) in vivo than in vitro following one dose. Deposition of an additional dose of caffeine

resulted in 19-fold more absorption than predicted by simple linear absorption. An additional dose in vitro demonstrates no enhanced absorption of the second dose. The enhanced absorption in vivo was unexpected, as viable, functional skin was anticipated to have better barrier properties than nonviable skin. Furthermore, it was noted that the total amount of caffeine absorbed in vitro was significantly less than that in vivo. These data suggest nonlinear absorption kinetics, most probably due to tissue binding (see below) and demonstrate the importance of basing conclusions relative to percutaneous absorption on in vivo analyses.

The foregoing suggested that caffeine absorbed in the in vivo state has only to cross the capillary bed where it is absorbed, whereas caffeine that is absorbed in vitro has to traverse the entire dermis before appearing in the receiver chamber. Traversing the entire dermis in vitro may result in more extensive tissue binding which would decrease the amount of caffeine entering the receiver chamber.

Further investigation of the possible tissue binding phenomenon was performed with 2-mm punch biopsies collected from the in vivo and in vitro model systems at the end of one- and two-dose experiments. Data revealed that tissue binding in vitro was more extensive than in vivo, 5-fold and 30-fold greater following one and two doses, respectively. Caffeine tissue binding in vivo remained the same following either one or two doses, yet flap blood concentration was enhanced 10-fold following two doses. Thus, the use of in vitro model systems may indeed be inadequate in reflecting the percutaneous absorption of a compound when tissue binding of that compound is a factor in that process.

Prior to the development of the skin sandwich flap system, it was difficult to actually assess metabolic activity of skin in situ. In vitro and in vivo transdermal absorption and metabolism of the antiviral compound, vidarabine (ara-A), and its deaminated metabolite, 9-beta-D-arabinofuranosyl-hypoxanthine (ara-H), were used to validate the utility of this system to assess metabolic capabilities of the skin in vivo. While these experiments revealed no difference between in vitro and in vivo metabolism of ara-A, they do not suggest that this is generally true for all compounds.

Feasibility of transplanting hair-bearing skin on nude rats

Throughout the above experimentation with human-rat skin sandwich flaps, it was observed that an increased amount of topically applied agent was absorbed when applied to skin rich in hair follicles (rat vs. human skin). While the number of terminal hairs in the nude rat is less than that of its littermates, it is at least 10-fold that of normal nonhair-bearing human skin. To date, there have been no descriptions of the hair growth following transplantation of human split-thickness scalp skin grafts on nude rats treated with cyclosporine that we report. This phenomenon was not observed in similar grafts on nude rats treated with Imuran and hydrocortisone or with antilymphocyte serum.

Two stages of hair growth in the split-thickness scalp grafts were noted, immediate and delayed. Hair growth in the immediate stage (6 weeks after engraftment) was more dense, occurring in the apparent absence of papillae, and was less tenaciously held within the graft than hair grown in the delayed stage. New papillae formation, as detected histologically, hair growth density

(hair/cm^2), and hair length (mm) were significantly greater ($p<0.001$) in the 1.0-mm scalp skin grafts than in the 0.4-mm grafts. The foregoing data suggest that split-thickness human scalp skin bearing adnexal structures has the ability to reestablish hair growth after transplantation to the nude rat. Furthermore, it is anticipated that differences in the percutaneous absorption of topically applied compounds between adnexal-rich and adnexal-poor skin can be accomplished with one system and transplants of this type.

To our knowledge, the human-rat skin sandwich flap is the first example of viable, functional human skin that is chronically maintained by a biologic support system and which has the added distinction of being supplied by an independent but accessible vasculature. The foregoing experiments strongly suggest that this system will be important in gaining insights into the more sophisticated percutaneous absorption processes of human skin. Additional features of this system which make it attractive as an experimental system include the ability to:

1) monitor systemic blood levels as a function of the local cutaneous blood concentration;
2) monitor the disappearance of the test compound from the donor chamber and therewith accurately determine transdermal flux;
3) measure the oxidative metabolism of compounds locally in the skin;
4) differentiate these features in a variety of skin sites, including abdomen, face, and scalp;
5) study the influence of temperature, both internal and external, and humidity on percutaneous absorption;
6) evaluate the delivery of single or multiple doses of orally administered drugs to the skin; and
7) evaluate the interaction of multiple drugs or toxic agents at the skin and the efficacy of preventative devices to decrease detrimental side effects from toxic agents.

Literature Cited

1. Scheuplein RJ, Bronaugh RL: Percutaneous absorption. In: Biophysical Properties of the Skin, Vol. 1, LA Goldsmith (ed), New York, Oxford University Press, pp. 1255-1295, 1983.
2. Reifenrath WG, Chellquist EM, Shipwash EA, Jederberg WW, Krueger GG: Percutaneous penetration in the hairless dog, weanling pig, and grafted athymic nude mouse: Evaluation of models for predicting skin penetration in man. Br J Dermatol (Suppl 27) 111:123-135, 1984.
3. Black KE, Jederberg WW: Athymic nude mice and human skin grafting. In: Models in Dermatology, Vol 1, H Maibach and N Lowe (eds), Basel, Karger, pp. 228-239, 1985.
4. Wojciechowski ZJ, Huether SE, Leonard LG, Krueger GG: Skin on an isolated accessible vasculature as a model system to study skin pathophysiology. Clin Res 32(2):482A, 1984.
5. Burton SA, Wojciechowski ZJ, Rohr U, Krueger GG, Higuchi WI: Comparison of in vivo and in vitro transdermal drug absorption and dermal drug metabolism of vidarabine using a unique isolated skin flap model. Clin Res 31(1):152A, 1985.
6. Wojciechowski ZJ, Burton SA, Petelenz TJ, Krueger GG: Role of microcirculation in percutaneous absorption. Clin Res 33(1):21A, 1985.
7. Huether SE, Wojciechowski ZJ, Petelenz TJ, Leonard LG, Krueger GG: A model for measuring skin blood flow. Clin Res 33(1):92A, 1985.
8. Gilhar A, Wojciechowski Z, Krueger G: A study of human skin grafts on congenitally athymic (nude) rats. Clin Res 33(1):22A, 1985.
9. Brooks CG, Webb PJ, Robins RA, Robinson G, Baldwin RW, Festing MRW: Studies on the immunobiology of rnu/rnu "nude" rats with congenital aplasia of the thymus. Eur J Immunol 10:58-67, 1980.
10. Vos JG, Kreeftenberg JG, Kruijt BC, Kruizinga W, Steerenberg PA: The athymic nude rat. II. Immunologic characteristics. Clin Immunol Immunopathol 15:229-236, 1980.
11. Krueger GG, Briggaman RA: The nude mouse in the biology and pathology of skin. In: The Nude Mouse in Experimental and Clinical Research, 2nd Edition, J Fogh and BC Giovanella (eds), New York, Academic Press, pp. 301-322, 1982.
12. Marks NJ: Quantitative analysis of skin flap blood flow in the rat using laser Doppler velocimetry. J Roy Soc Med 78:308-312, 1985.
13. Holloway EA, Watkins DW: Laser Doppler measurement of cutaneous blood flow. J Invest Dermatol 69:306-309, 1977.
14. Watkins DW, Holloway GA: An instrument to measure cutaneous blood flow using the Doppler shift of laser light. I.F.F.F. Trans Biomed Eng 25:28-38, 1978.
15. Silverman DG, La Rossa DD, Barlor CH, Bering GB, Popky LM, Smith TC: Quantification of tissue fluorescein delivery and prediction of flap viability with the fiberoptic dermofluorometer. Plast Reconstr Surg 66:545-551, 1980.
16. Graham GH, Walton RL, Elings VB, Lewis FR: Surface quantification of injected fluorescein as a predictor of flap viability. Plast Reconstr Surg 71:826-832, 1983.
17. Reinisch JF: The pathophysiology of skin flap circulation; the delay phenomenon. Plast Reconstr Surg 54:585-598, 1974.
18. Marks NJ, Trachy RE, Cummings CW: Dynamic variations in blood flow as measured by laser Doppler velocimetry: A study in rat skin flap. Plast Reconstr Surg 73:804-810, 1984.

19. Petry JJ, Wortham KA: The anatomy of the epigastric flap in the experimental rat. Plast Reconstr Surg 74:410-415, 1984.
20. Finseth F, Cutting G: An experimental neurovascular island skin flap for study of the delay phenomenon. Plast Reconstr Surg 61:412-420, 1978.
21. Sasaki GH, Pang CY: Hemodynamics and viability of acute neurovascular island skin flaps in rats. Plast Reconstr Surg 65:152-159, 1980.
22. Scheuplein RJ, Ross LW: Mechanism of percutaneous absorption vs. percutaneous absorption of solvent-deposited solids. J Invest Dermatol 62:353-360, 1974.
23. Feldmann RJ, Maibach HI: Absorption of some organic compounds through the skin in man. J Invest Dermatol 54:390-394, 1970.
24. Davies DAL, Staines NA: A cardinal role for I region antigens (Ia) in immunological enhancement, and the clinical implications. Transplant Rev 30:18, 1976.
25. Hefton JM, Amberson JB, Biozes DG, Weksler ME: Loss of HLA-DR expression of human epidermal cells after growth in culture. J Invest Dermatol 83:48, 1984.
26. Ediger R, Giovanella BC: Current knowledge of breeding and mass production of the nude mouse. In: The Nude Mouse in Experimental and Clinical Research, Vol. 1, J Fogh and BC Giovanella (eds), New York, Academic Press, p. 16, 1978.
27. Barlozzari T, Reynolds CW, Herberman RB: In vivo role of natural killer cells: Involvement of large granular lymphocytes in the clearance of tumor cells in anti-asialo GM treated rats. J Immunol 13:1024-1027, 1983.
28. Pinto M, Gill T[illegible], Klunz HW: Prolongation of skin allograft survival across different genetic barriers in rats with cyclosporine, and its potentiation of Bordetella pertussis vaccine. Transplantation 36:171-176, 1983.
29. Headington JT: Transverse microscopic anatomy of the human scalp: A basis for a morphometric approach to disorders of the hair follicles. Arch Dermatol 120:449-456, 1984.
30. Chu AC, Patterson YAK, Goldstein G, Berger CL, Takezaki S, Edelson RL: Thymopoietin-like substance in human skin. J Invest Dermatol 81:194-197, 1983.
31. Spangrude GJ, Bernhard EJ, Ajioka RS, Daynes RA: Alterations in lymphocyte homing patterns within mice exposed to ultraviolet radiation. J Immunol 130:2974-2981, 1983.
32. Bach FJ, Bach ML, Sondel PM: Differential function of major histocompatibility complex antigens in T-lymphocyte activation. Nature 259:273, 1976.
33. Meo T, David CS, Riynbeek AM, Mabholz M, Miggiano VC, Shreffler DC: Inhibition of mouse MLR by anti-Ia sera. Transplant Proc 7:127, 1975.
34. Festing MFW, May D, Connors TA, Lovell D, Sparrow S: An athymic nude mutation in the rat. Nature 274:365-366, 1978.
35. Lotzova E, Savary CA, Stringfellow DA, Drewinka B, Gray KW, Raulston GL, Jardine JH: Analysis of natural killer cell activity in random-bred Rowett athymic rats. In: Immunodeficient Animals in Experimental Research, B Sordat (ed), Basel, Karger, pp. 53-59, 1984.
36. Kindred B: The nude mouse in studying T-cell differentiation. In: The Nude Mouse in Experimental and Clinical Research, J Fogh and BC Giovanella (eds), New York, Academic Press, pp. 111-134, 1978.
37. Bazin H, Platteau B, Pauwels R, Carpon A: Immunoglobulin production in nude rats with special attention to the IgE isotope. Ann Immunol (Inst Pasteur) 131C:31-37, 1980.

38. Lear AP, Heidecke CD, Kupiec-Wegliuski YW, Acaneda D, Strom TB, Tieney NL: Restoration of allograft responsiveness in B rats. Transplantation 36:412-417, 1983.
39. Sauder DN, Carter CS, Katz SI, Oppenheim JJ: Epidermal cell production of thymocyte activating factor (ETAF). J Invest Dermatol 79:34-39, 1982.
40. Douglas FW, Ting JP, Frelinger JA: Ia antigens on non-lymphoid tissues. Their origin and functions. Immunol Today 12:339, 1982.
41. Steinmuller D: Passenger leukocytes and the immunogenicity of skin allograft. Transplant Proc 13:1094, 1981.
42. Morison WL, Parrish JA, Woehler ME, Bloch KJ: The influence of PUVA and UVB radiation on skin graft survival in rabbits. J Invest Dermatol 75:331-333, 1980.
43. de Waal RMM, Bogman MJJ, Maass CN, Cornelissen LMW, Taxs WJM, Koene RAP: Variable expression of Ia antigens on the vascular endothelium of mouse skin allografts. Nature 303:426, 1983.
44. Pinto M, Brent L, Thomas AV: Specific unresponsiveness to skin allografts in mice. III. Synergistic effect of tissue extracts, Bordetella pertussis and antilymphocytic serum. Transplantation 17:477-486, 1974.
45. Biren C, Barr R, McCullough J, Black K, Hewitt C: Prolonged viability of human skin xenografts in rats by cyclosporine (CsA). J Invest Dermatol 82: 438A (1985).
46. Franz TJ: Percutaneous absorption. On the relevance of in vitro data. J Invest Dermatol 64:190-195, 1975.
47. Homan WP, Fabre JW, Millard PR, Morris PJ: Effect of cyclosporin A upon second-set rejection of rat renal allografts. Transplantation 30:354-357, 1980.
48. Roskos KV, Hinz RS, Maibach HI, Guy RH: Effect of aging on percutaneous drug penetration in vivo. J Pharm Sci 74:P151, 1985.

Distribution List

1 copy: Commander
US Army Medical Research and Development Command
ATTN: SGRD-RMI-S
Fort Detrick
Frederick, MD 21701-5012

5 copies: Commander
US Army Medical Research and Development Command
ATTN: SGRD-PLE
Fort Detrick
Frederick, MD 21701-5012

12 copies: Administrator
Defense Technical Information Center
ATTN: DTIC-DDA
Cameron Station
Alexandria, VA 22304-6145

1 copy: Commandant
Academy of Health Sciences, US Army
ATTN: AHS-CDM
Fort Sam Houston, TX 78234-6100

1 copy: Dean, School of Medicine
Uniformed Services University of the Health Sciences
4301 Jones Bridge Road
Bethesda, MD 20814-4799